Congenital adrenal hyperplasia:

clinical and biochemical consequences of elevated adrenal steroid precursors

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Contents

Chapter 1

General introduction, aim and outline of the thesis

Steroid synthesis in the adrenal gland

The adrenal glands, though small, are essential for life. They are located on top of the kidneys and are composed of a cortex and a medulla. Steroid synthesis takes place in the adrenal cortex. The adrenal cortex consists of three anatomically distinct zones, where several steroid hormones are synthesized from the common precursor cholesterol: aldosterone in the outer zona glomerulosa, cortisol in the central zona fasciculata, and adrenal androgens in the inner zona reticularis. [1] Multiple enzymatic steps are required for adrenal steroid synthesis (figure 1).

Cortisol, a glucocorticoid hormone, is also known as a stress hormone. It regulates cardiovascular, metabolic and immune homeostasis. The production of cortisol is regulated through the hypothalamic – pituitary – adrenal (HPA) axis. The hypothalamus produces corticotrophin releasing hormone (CRH), which stimulates the release of adrenocorticotropic hormone (ACTH) by the anterior pituitary gland. ACTH promotes the conversion of cholesterol to cortisol in the adrenal cortex. The secretion of CRH and ACTH is controlled by three mechanisms: a negative feedback mechanism by cortisol, a circadian rhythm and stress-responsiveness of the HPA-axis. [1]

Aldosterone, a mineralocorticoid hormone, is involved in the water and salt homeostasis and primarily regulates blood volume. Aldosterone promotes the sodium reabsorption and potassium excretion in the kidneys. With the reabsorption of sodium, water is also retained. The secretion of aldosterone is mainly regulated through the renin – angiotensin – aldosterone system.

Adrenal androgens have weak androgenic activity and are peripherally converted to the more potent androgens testosterone and dihydrotestosterone. In adult men, most androgens are derived from the testes. However, in both males and females adrenal androgens are involved in pubertal development, and in females adrenal androgens contribute substantially to total androgen production and effect. Amongst other effects, androgens promote bone maturation and skeletal growth, and are necessary for adequate bone health. [2]

Congenital adrenal hyperplasia

Enzyme deficiency

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders of the adrenal cortex in which a deficiency of one of the enzymes involved in steroid synthesis results in disturbed steroidogenesis. The most common form,

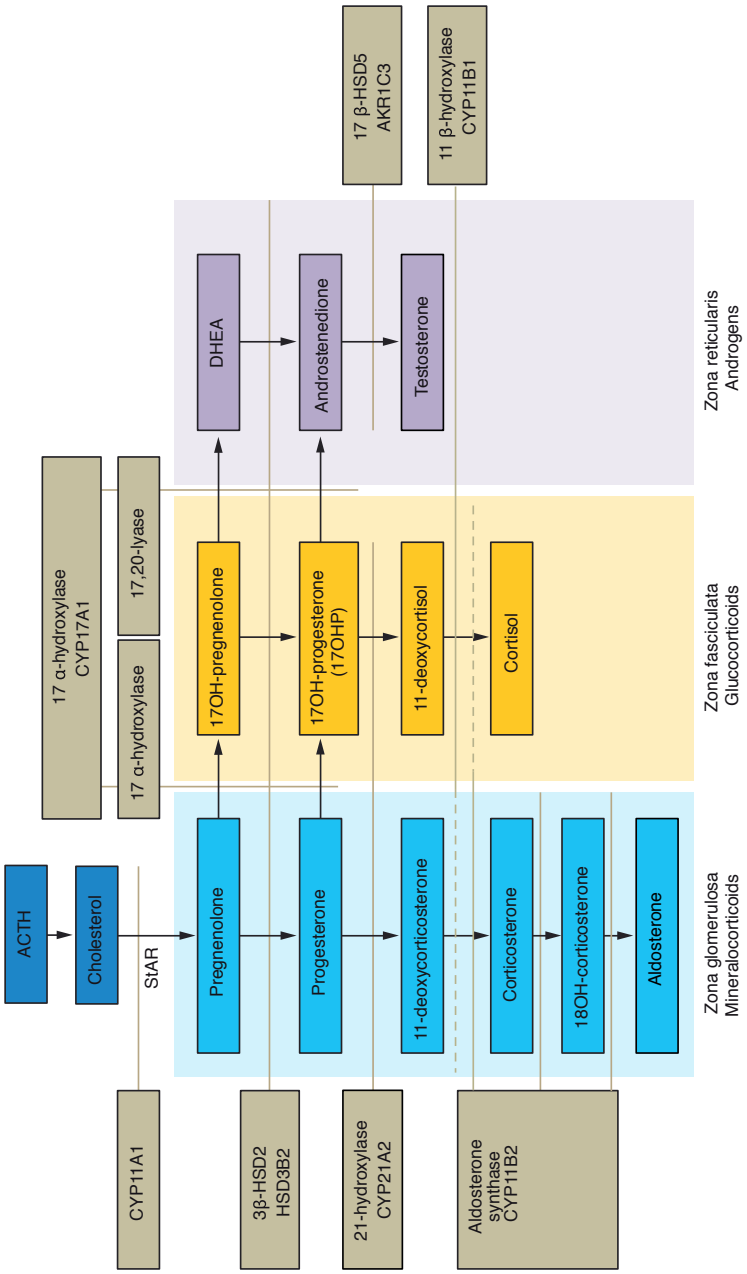

Figure 1 Normal adrenal steroid synthesis.

Adapted from Han et al, Nat Rev End 2014(10):115-124, with permission.

accounting for 95% of the cases, is 21-hydroxylase deficiency, caused by mutations in the *CYP21A2* gene. [3, 4] The incidence is approximately 1:12,000 in the Netherlands and other European countries. [5] The second most frequent type of CAH in European populations is 11-hydroxylase deficiency. Less common forms are deficiencies of StAR, P450scc, 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase and POR. [6, 7] This thesis will mainly focus on 21-hydroxylase deficiency.

In 21-hydroxylase deficiency, the conversion of 17-hydroxyprogesterone (17OHP) and progesterone to 11-deoxycortisol and 11-deoxycorticosterone respectively is impaired (figure 2). This results in a deficiency of cortisol and – in the most severe form – also of aldosterone. Due to the lack of negative feedback from cortisol to the hypothalamus and the pituitary gland, ACTH secretion is increased. The excess ACTH stimulation results in adrenal hyperplasia and accumulation of the steroid precursors proximal to the enzymatic block, mainly 17OHP and progesterone. These precursors are shunted into the androgen-synthesis pathway that does not require 21-hydroxylation, increasing the production of adrenal androgens. [3] Additionally, 17OHP is converted by alternative pathways. 11-hydroxylation of 17OHP results in the formation of 21-deoxycortisol. Also, through the so-called 'back-door pathway' dihydrotestosterone is formed from 17OHP without androstenedione or testosterone as intermediate steroids. [8]

Subtypes of CAH

Conventionally, CAH due to 21-hydroxylase deficiency is divided in subtypes depending on the remaining enzyme activity: a classic form usually diagnosed in the neonatal period, consisting of a salt wasting (SW) and a simple virilizing (SV) subtype, and a late onset or nonclassic (NC) form. [3]

SW-CAH is the most severe form of 21-hydroxylase deficient CAH, with a remaining enzyme activity of <1% resulting in deficiencies of both cortisol and aldosterone. The aldosterone deficiency leads to severe salt loss after the first week of life, which can result in life-threatening hyponatremia, hyperkalemia and dehydration. The cortisol deficiency can lead to adrenal crises, with vomiting, hypoglycemia, hypotension, and decreased consciousness. The androgen overproduction is already present antenatally and can result in various degrees of virilization of the external genitalia in females. In boys, external genital development is normal apart from occasionally hyperpigmentation. [3]

In SV-CAH, the residual enzyme activity is 1-5%. Cortisol synthesis is deficient, but aldosterone production is sufficient to prevent salt wasting crises. Androgen overproduction is present as in SW-CAH, resulting in antenatal virilization of female

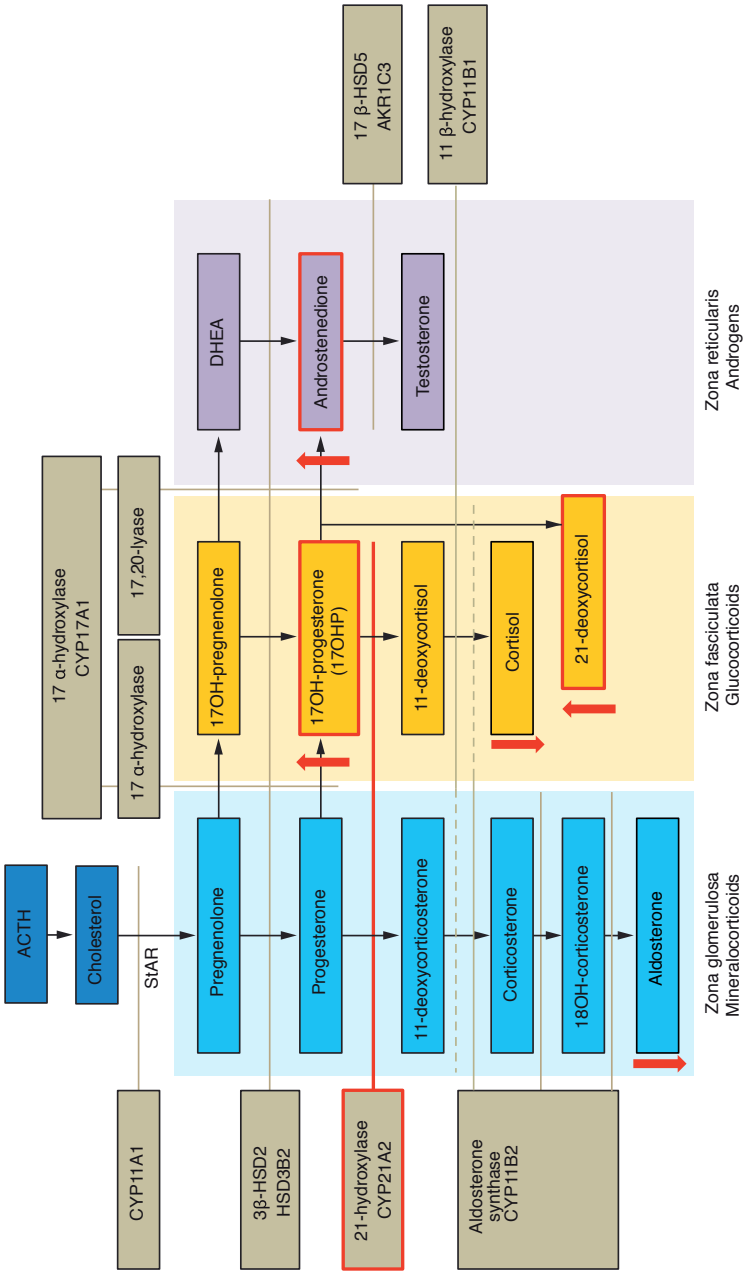

Figure 2 Adrenal steroid synthesis in 21-hydroxylase deficiency.

Adapted from Han et al, Nat Rev End 2014(10):115-124, with permission.

external genitalia and signs of androgen excess such as precocious pseudopuberty occurring in male patients in the first years of life. [7]

The NC form of CAH is less severe with an enzyme activity of 20-50%. Aldosterone production is sufficient. Baseline cortisol levels are not reduced, although ACTH stimulated cortisol levels may be decreased. [9, 10] Androgens are slightly elevated. Clinical signs and symptoms of androgen overproduction can occur later in life, for example precocious pseudopuberty in childhood; acne, hirsutism and menstrual disturbances in adolescence and adulthood; and subfertility in adulthood. However, not all individuals with genotypes consistent with NC-CAH develop clinical signs and symptoms. [3, 9, 11, 12]

Nowadays, CAH is considered to be a spectrum of disease, depending on the severity of the underlying *CYP21A2* mutation and the residual activity of 21-hydroxylase. In clinical practice the distinction between the conventional subtypes is not always clear.

Genetics

The *CYP21A2* gene is located at chromosome 6p21.3 and lies in close proximity to a highly homologous pseudogene *CYP21A1P*. Over 200 mutations in the *CYP21A2* gene have been described, however approximately ten mutations account for most affected alleles (figure 3) (www.pharmvar.org/gene/CYP21A2). [13] Most mutations result from non-homologous recombination or gene conversion events with the pesudogene. [14]

The *CYP21A2* mutations have been categorized in mutation groups based on their *in vitro* 21-hydroxylase activity, in analogy with the clinical subtypes. [15] In mutation group null, there is no residual enzyme activity. In groups A, B and C the residual enzyme activity is 0-1%, 1-5% and 20-50% respectively. Groups null and A are usually associated with a SW phenotype, group B with a SV phenotype and group C with a NC phenotype. Most CAH patients are compound heterozygotes with different mutations on each allele. The phenotype is usually determined by the least severe mutation of the two affected alleles. [7, 11] The genotype-phenotype correlation is generally good, however exceptions have been found and some mutations can result in various different phenotypes. [3, 14]

Diagnosis

The diagnosis of CAH can be made biochemically, by determining the levels of adrenal steroid precursors (in 21-hydroxylase deficiency most importantly 17OHP) by immunoassays and – preferably – mass spectrometry methods, because of

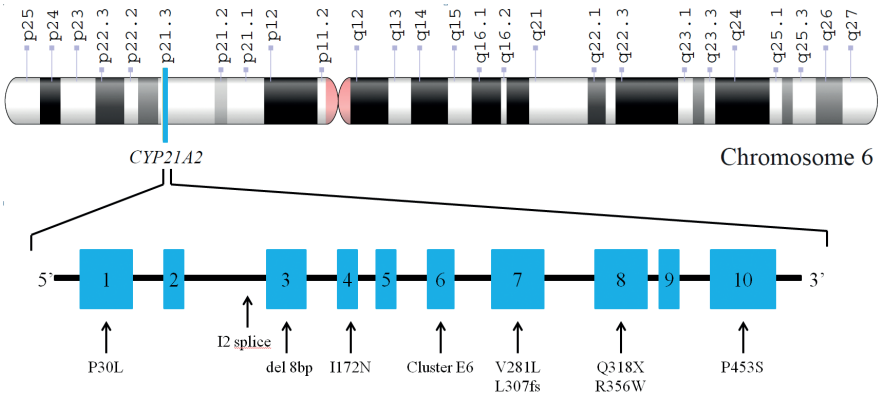

Figure 3 The location of the *CYP21A2* gene on the short arm of chromosome 6.

The blue boxes represent the exons of the *CYP21A2* gene. The most frequently encountered mutations in 21-hydroxylase deficiency – other than large deletions – are depicted. The I2 splice mutation is located in an intron.

the risk of cross reactivity with other steroid metabolites in immunoassays. ACTH stimulation tests can be required. The diagnosis can be confirmed by mutation analysis. [3, 4, 11]

Historically, CAH was diagnosed after the occurrence of clinical signs and symptoms such as ambiguous genitalia at birth and/or salt wasting crises in the neonatal period. Since the year 2002, CAH is included in the neonatal screening program in the Netherlands. In a dried filter paper blood spot collected within 72-96 hours after birth, the level of 17OHP is determined. When it is above the cut-off values for gestational age, the patient is immediately referred to a pediatric endocrinology centre for a further diagnostic work-up. This ensures early diagnosis and aims to prevent the occurrence of severe salt wasting crises especially in males, in whom no signs of excess androgen exposure are apparent at birth. Evaluation of the screening program has shown that more than 30% of CAH girls are also primarily diagnosed through the neonatal screening program, as virilization of the external genitalia can be overlooked. [5] Both SW-CAH and SV-CAH are detected in the neonatal screening program, while NC-CAH is normally not diagnosed since the 17OHP concentration is generally below the cut-off level in this mild form of CAH.

Treatment and treatment goals

The treatment of classic CAH consists of chronic glucocorticoid and if necessary also mineralocorticoid therapy. The aim of the treatment is not only to replace the deficient hormones, but also to suppress the excess production of adrenal androgens. Through the treatment with glucocorticoids, the negative feedback to the hypothalamus and pituitary is restored. The ACTH secretion is suppressed, resulting in less stimulation of the adrenal cortex and less production of adrenal androgens. [3]

Specific treatment goals differ according to the patient's age. In childhood and adolescence, main goals are to ensure normal linear growth and skeletal maturation, to prevent early pubertal development and to prevent symptoms of hyperandrogenism. In adult patients, maintaining fertility is an important objective. In all patients, ensuring optimal quality of life and avoiding the long-term consequences of chronic glucocorticoid use are main issues. [11, 16, 17]

To reach these treatment goals, the balance between over- and undertreatment needs to be found. To adequately suppress adrenal androgens, often supraphysiological doses of glucocorticoids are necessary. [4, 11] Failure to suppress adrenal androgens can lead to increased growth velocity, but also premature epiphyseal maturation ultimately resulting in a decreased final height. Also, during puberty and adulthood gonadal function can be impaired since elevated androgens, which are converted to estrogens, can suppress gonadotropin secretion by the pituitary gland. [4, 9] However, chronic use of supraphysiological doses of glucocorticoids can result in side effects such as hypertension, insulin resistance, obesity and a decreased bone mineral density. It can also result in a decreased final height, due to direct effects on the growth plate and impaired growth hormone secretion and action. [3, 4, 7, 9, 18] Therefore, adequate linear growth and final height can serve as long-term outcome parameters of the treatment of CAH in children.

To ensure adequate treatment of this complex disorder, patients should be monitored closely throughout their lives using both clinical and laboratory parameters. In childhood and adolescence, regular measurements of height, weight, blood pressure, bone age, adrenal steroid precursor levels (usually 17OHP and androstenedione) and renin levels should be performed. [4, 17] During treatment, the glucocorticoid dose needs to be increased in situations of stress such as significant illness, trauma and surgery to prevent the occurrence of adrenal crises. Parenteral administration of glucocorticoids may be necessary in these situations. An important part of the care of CAH patients is appropriate education of the patients and their parents on when and how to apply stress-dosing and this education should be repeated during follow-up. [19]

For NC-CAH patients, treatment should focus on the patient's symptoms, not merely on normalizing abnormal steroid hormone levels. It is advised to reserve chronic glucocorticoid treatment for patients with clinically significant signs of hyperandrogenism. [3, 4, 7, 9, 11, 12, 17] There is still debate on the need for stress-dosing in NC-CAH patients who are not on chronic glucocorticoid therapy. Although ACTH stimulated cortisol levels may be decreased, clinical manifestations of adrenal insufficiency seem uncommon in NC-CAH patients. [10] Routine stress dosing is currently not recommended, but it may be advisable during serious illness or major surgery in patients with an inadequate cortisol response after ACTH administration. [7, 9, 10, 12, 17]

Aim and outline of the thesis

Despite the increasing understanding of CAH, many questions remain unanswered. This thesis aims to gain more insight in the pathophysiological processes related to elevated adrenal steroid precursors, both from a clinical and from a biochemical perspective.

The first part of the thesis contains clinical studies, focusing on growth patterns and adrenal steroid levels in CAH patients. These are important elements of the clinical evaluation of CAH patients, both in diagnosis and in long term follow-up. In **chapter 2**, we describe the patterns of linear growth and bone maturation of untreated children with NC-CAH. In **chapter 3**, we focus on the salivary levels of 17OHP and androstenedione in treated children with classic CAH, before and during puberty. **Chapter 4** describes the long term follow-up of classic CAH children, combining their patterns of linear growth and bone maturation with longitudinal measurements of their salivary steroid measurements and glucocorticoid use.

In the second part of this thesis the glucocorticoid and mineralocorticoid potency of adrenal steroid precursors is examined. The starting point is the description of an untreated SW-CAH patient in **chapter 5**, who showed no clear signs of cortisol deficiency. Combined with other clinical observations, this has led to the hypothesis that the accumulating adrenal steroid precursors may have glucocorticoid properties. In **chapter 6** we evaluate the *in vitro* binding and transactivation of the human glucocorticoid receptor (hGR) by the adrenal steroid precursors 21-deoxycortisol, 17OHP, progesterone and androstenedione. In **chapter 7**, we expanded the *in vitro* transactivation studies of the hGR with additional adrenal steroid precursors. Additionally, in this chapter we describe a unique cohort of untreated classic CAH patients with clinical data on episodes of severe stress they survived without

glucocorticoid treatment, and with biochemical data on adrenal steroid levels. **Chapter 8** focuses on the *in vitro* binding and transactivation of adrenal steroid precursors to the mineralocorticoid receptor.

In the concluding part of this thesis, **chapter 9**, the results and conclusions of the thesis are discussed in relation to the existing literature. Future perspectives are explored. **Chapter 10** consists of a summary of the studies included in this thesis. The **addendum** contains the Dutch summary as well as a list of abbreviations, list of publications, acknowledgements and curriculum vitae.

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Part I

Patterns of growth and salivary steroid levels in congenital adrenal hyperplasia patients

Chapter 2

Absence of clinically relevant growth acceleration in untreated children with nonclassic congenital adrenal hyperplasia

Pijnenburg-Kleizen KJ, Borm GF, Otten BJ, Schott DA,
van den Akker EL, Stokvis-Brantsma WH, Voorhoeve PG, Bakker B,
Claahsen-van der Grinten HL. Horm Res Ped 2012; 77(3): 164-9

Abstract

Background/Aims In classic congenital adrenal hyperplasia (CAH) elevation of adrenal androgens leads to accelerated growth and bone maturation with compromised adult height. In untreated children with nonclassic CAH (NC-CAH), in which adrenal androgens are generally only slightly increased, growth velocity may not be significantly elevated.

Methods 24 patients were included and divided in a symptomatic and an asymptomatic group. Height was expressed as height standard deviation score (HSDS) and corrected for target height (HSDS-THSDS). Bone maturation was expressed as bone age acceleration (BA_C= bone age - calendar age). Linear mixed models with random factor patient were used for the analysis of growth and bone age.

Results In symptomatic patients (n=17) HSDS-THSDS only slightly increased with 0.06 SDS per year (95% CI 0.02 – 0.10). Mean BA_C was 2.21 years (SDS 0.66, $p< 0.0001$). In asymptomatic patients (n=7) no significant growth acceleration or BA_C was found.

Conclusions In untreated NC-CAH children growth acceleration is small and generally not visible in their growth charts. BA_C is more pronounced. Therefore, the absence of an increase in growth velocity does not exclude the diagnosis NC-CAH. When considering this diagnosis, bone age acceleration should also be taken into account.

Introduction

Congenital adrenal hyperplasia (CAH) is one of the most common autosomal recessive endocrine disorders. It is characterized by specific enzymatic defects in the steroid biosynthesis. In most cases the deficient enzyme is 21-hydroxylase (21OHD) leading to impaired production of cortisol and in classic salt wasting (SW) cases also of aldosterone. As a consequence of impaired cortisol synthesis the secretion of ACTH by the pituitary gland is increased, leading to hyperplasia of the adrenal cortex. Steroid precursors immediately proximal to the defective step accumulate and are shunted into the androgen pathway, resulting in excessive production of adrenal androgens.

Three forms of 21OHD CAH can be distinguished by clinical, hormonal and molecular genetic criteria: the classic SW, classic simple virilising (SV), and nonclassic (NC) form.

In the most severe, SW form, which has an incidence of 1:20.000 [1], the residual enzymatic activity is less than 1% leading to cortisol deficiency as well as decreased aldosterone synthesis. Female patients are virilised prenatally due to adrenal androgen excess and neonates of both sexes can suffer from life-threatening SW. In classic SV-CAH, with an incidence of 1:60.000 [1], the residual enzymatic activity is 1-5%. Aldosterone synthesis is usually intact, but cortisol synthesis is impaired. Affected females present with genital ambiguity.

The mildest, NC form of CAH, or late onset type, is more common: the incidence is 1:1000 [1], It is characterized by a residual enzymatic activity of 20-50% with generally normal cortisol and aldosterone production but with mild androgen excess leading to variable signs of hyperandrogenism that can manifest at any age. [2-5] The main symptoms in childhood and adolescence include premature pubarche, cystic acne, hirsutism, and menstrual disorders. [1-8]

In patients with classic CAH androgen excess leads to a progressive increase in growth velocity and bone maturation after the first year of life. [9] Therefore, adequate suppression of adrenal androgens is important to prevent early epiphyseal fusion and compromised adult height. In children with NC-CAH adrenal androgens are only slightly elevated. Whether growth is accelerated in childhood and adult height is compromised in NC-CAH is not yet agreed upon. [1,2,6,7,10-12] A study describing the phenotypic profile of parents of CAH children, who were identified by family genetic studies as having NC-CAH, showed adult heights in the normal range. However, these parents were either asymptomatic or developed symptoms in adulthood. Therefore, their growth pattern might have been different from the growth pattern of children who are already symptomatic. [13]

Based on our personal clinical observations, we hypothesize that in children with NC-CAH growth velocity may not be significantly elevated at the time of diagnosis. We therefore retrospectively evaluated a group of children with NC-CAH to define their growth pattern prior to treatment. Furthermore, we evaluated the age of onset of symptoms, the presenting symptoms and the bone maturation.

Patients and Methods

Patients

We collected the data of NC-CAH children treated or followed at the Radboud University Nijmegen Medical Center from 1980 until 2009, and of patients currently under treatment or follow-up in five other Dutch hospitals (3 University Hospitals). The diagnosis of NC-CAH was based on clinical and biochemical criteria and was confirmed by mutation analysis.

Age at diagnosis, clinical symptoms at presentation, bone age and growth data from birth until initiation of treatment were obtained from the patient's medical records. In most cases adult heights were not available. Those that were available were not collected, since they could be affected by treatment and therefore do not only represent the effect of androgen excess on growth in this population. Height was expressed as height standard deviation scores (HSDS) according to the national references [14] and corrected for target height, defined as corrected midparental height (HSDS-THSDS). [9] Bone maturation (bone age, BA) was determined according to Greulich and Pyle and expressed as advance in bone age (BA_C= BA – chronological age).

Statistical analysis

Data were analysed using Statistical Package for Social Sciences (SPSS) for Windows (version 16.0). The growth and bone age were analyzed for the entire patient group and for two subgroups separately. The first subgroup (symptomatic group) consists of patients diagnosed after the onset of signs or symptoms of increased androgens; the second subgroup (asymptomatic group) concerns children, who did not show signs or symptoms but were detected by family screening or the neonatal screening program. This division was made because of the fact that the latter group may not develop signs of androgen overproduction until adulthood and their growth patterns may be different from those of the patients who become symptomatic in childhood.

Since in the first years of life growth patterns can be different from later years due to target seeking, and in the initial analysis the growth data of symptomatic patients showed a different pattern before and after the age of three years, separate analyses were done for the age groups 0-3 years and >3 years. Linear mixed models with random factor patient were used for the analysis of the growth and the bone age. Ninety-five percent confidence intervals (CI) were calculated.

Results

Clinical presentation

24 NC-CAH patients were included, 14 females and 10 males (Table 1). 17 patients were included in the symptomatic group; their mean age at presentation was 7.1 years (range 2.4 – 9.3 years). The main presenting symptom was precocious

Table 1 Clinical presentation and BAc at presentation, and mutation analysis in 24 patients with NC-CAH.

Patient No.	Sex	Age at presentation (yrs)	Symptoms at presentation[a]	BA_c[b] at presentation (yrs)	Mutation analysis	
					Allele 1	Allele 2
Symptomatic patients						
1	F	2.4	3	-0.1	656A/C>G	P453S
2	F	3.0	1	-	V281L	V281L
3	F	4.6	1,3	-	R356W	902C>A
4	M	7.3	1	3.5	V281L	Del-Conv
5	F	8.7	1,3	2.3	V281L	8BP-DEL
6	F	6.3	1	-	V281L	R408C
7	M	7.8	1	4.7	V281L	656A/C>G
8	F	7.4	1,2	1.3	V281L	V281L
9	M	8.5	1	-0.1	V281L	Del/Conv
10	F	9.3	1	2.2	V281L	R356W
11	F	8.3	1,3	1.9	V281L	P30L
12	M	7.4	1	2.7	V281L	656A/C>G
13	F	7.0	1,2	3.0	V281L	V281L
14	F	7.0	1	3.1	V281L	656A/C>G
15	F	9.0	1,2	1.0	Del-Conv	P432L
16	F	8.0	1	1.7	I172N	1177C>T
17	M	8.3	1	3.2	P453S	Del-Conv
Asymptomatic patients						
18	M	0.0	4	0.6	V281L	I172N
19	M	8.3	4	-0.5	V281L	V281L
20	F	4.5	4	1.0	V281L	R356W
21	F	6.2	4	0.6	V281L	Del-Conv
22	M	0.0	4	-0.2	V281L	Del-Conv
23	M	3.0	4	0.5	V281L	656A/C>G
24	M	6.0	4	1.3	P453S	Del-Conv

[a] Symptoms at presentation. 1, pubic hair; 2, axillary hair; 3, clitoromegaly; 4, asymptomatic
[b] BA_c Bone age – Chronological age

pubarche. Treatment was started in all but one 7-year-old female patient, presenting recently with precocious pubarche. Mean age at initiation of treatment was 8.1 years (range 2.5 – 12.3 years), mean 0.9 years after first presentation (range 0.1 – 2.0 years).

7 patients were included in the asymptomatic subgroup. They were diagnosed because of an affected sibling (n=6) or because of a positive CAH screening in the national screening programme (n=1). Their mean age at presentation was 4.0 years (range 0.0 – 8.3 years). All patients were followed in outpatient clinics to monitor them for signs and symptoms of androgen excess. In two initially asymptomatic patients treatment was started at the age of 7.5 and 8.1 years, because of an increasing advance in bone age, respectively 1.3 and 8.1 years after diagnosis.

Growth analysis

In the entire patient group HSDS-THSDS increased with 0.06 SDS per year (95% CI 0.03 – 0.09, p = 0.003) (Figure 1).

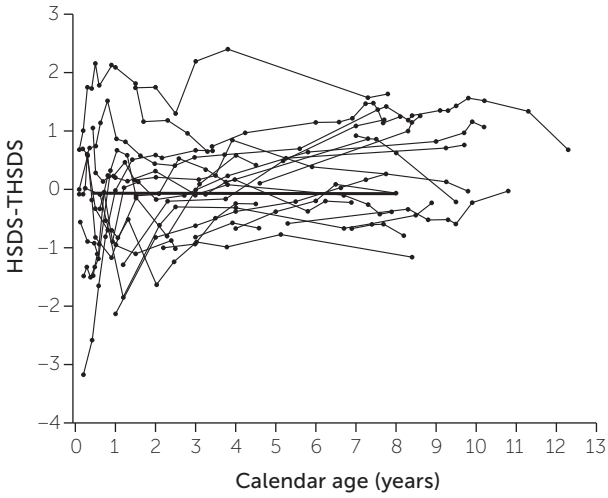

Figure 1 Growth expressed as HSDS-THSDS for chronological age in 24 NC-CAH patients (14 symptomatic patients, 7 asymptomatic patients).

The thick line represents the regression coefficient (0.06 SDS per year, 95% CI 0.03 – 0.09, p = 0.003).

In the symptomatic group growth data were available from 0 to 12.3 years of age. The mean number of measurements per patient was 8.4 (range 3 – 16). In the first three years of life HSDS-THSDS increased with 0.33 SDS per year (95% CI 0.08 -

0.59, p = 0.01), with a large variation in growth velocity. After the age of three years there was a small increase in HSDS-THSDS of 0.06 SDS per year (95% CI 0.02 – 0.10, p = 0.002).

In the asymptomatic group, growth data were available from 0 to 9.0 years of age. The mean number of measurements per patient was 10.6 (range 5 – 15). No growth acceleration was seen in this patient group, with a mean HSDS-THSDS of -0.07 (SDS 0.74, p = 0.80).

Bone age analysis

In 19 patients bone age was determined at presentation. In 11 patients bone age determination was repeated once or twice during follow-up. BA_C increased with 0.11 years per year (95% CI -0.01 – 0.24, p=0.08) (Figure 2). Measurements at a younger age (0-7 years) were almost exclusively of the asymptomatic patients, measurements at an age of 7 years and older were mainly of symptomatic patients. In symptomatic patients mean BA_C was 2.21 years (SDS 0.66, p < 0.0001). Analysis of BA_C of asymptomatic patients showed a - not statistically significant - mean advance of 0.45 years (SDS 0.66, p = 0.15) Because of the limited number of measurements and the relatively small time intervals it could not be determined whether there is an increase in BA_C over the course of time in the two subgroups.

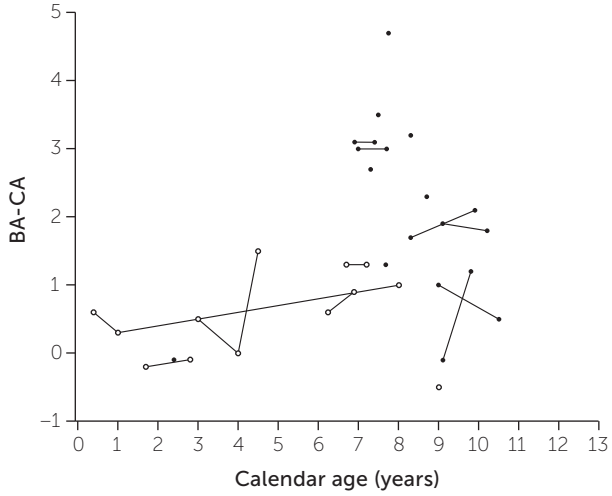

Figure 2 Advance in bone maturation expressed as Bone age – Chronological age (BA_C) in years in 13 symptomatic NC-CAH patients (closed dots) and in 6 asymptomatic NC-CAH patients (open dots).

Discussion

To our best knowledge this is the first report describing the growth pattern and bone maturation of symptomatic and asymptomatic untreated children with NC-CAH. In our study group the growth acceleration was very small even in symptomatic children (0.06 SDS per year), most likely not clinically recognizable in the growth charts. Despite this absence of relevant growth acceleration we found an increase in BA_C over time and a significant BA_C at the age of 7-11 years in the symptomatic patient group.

Increased growth velocity is viewed as one of the most prominent symptoms of androgen excess in childhood. In prepubertal classic CAH patients overproduction of adrenal androgens leads to accelerated growth velocity and bone maturation with compromised adult height. [9,12,15-17] Treatment with glucocorticoids aims to suppress the androgen secretion, and to restore normal growth and maturation. However, oversuppression can lead to Cushing syndrome and growth retardation. [1,11,12,15-18] A recent meta-analysis describing the adult height of classic CAH patients who started glucocorticoid treatment in the first 5 years of life, showed that the final height is still decreased, though in the normal range: -1,38 SDS. [19] Factors that positively influence the final height are a young age at diagnosis and good compliance with therapy. [12]

Skeletal growth and maturation is a complicated process depending not only on androgens but also on estrogens and other growth factors. Testosterone can stimulate growth directly through the androgen receptor in osteoblasts, osteoclasts, osteocytes and growth plate chondrocytes. [20] However, evidence of recent years indicates that the growth promoting effects of testosterone are mainly mediated through aromatization to estrogens. In male patients suffering from either an estrogen deficiency or estrogen resistance skeletal development is significantly delayed, suggesting a dominant role of estrogens in bone maturation. [20-22] Besides their direct effect on skeletal growth, androgens and estrogens may also influence the growth hormone – insulin-like growth factor I axis. [20,21]

The sensitivity to androgens seems to differ in different age groups. In an earlier study describing the growth patterns of untreated children with the classic SV form of CAH we showed that growth velocity and bone maturation are not increased in the first year of life [9], suggesting a relative resistance to androgen excess at this age, due to a reduced amount of receptors or to post-receptor effects. Thereafter, androgen excess leads to a progressive increase in growth velocity and bone maturation in strong relation to the duration of androgen exposure. [9] Therefore, after the age of 1 year growth velocity and bone maturation are valuable and well

known markers for the diagnosis and follow up of children with significant androgen excess syndromes.

Our results suggest that the response to androgens is not only age-dependent but also dose-dependent: in untreated NC-CAH children with generally only mild androgen excess, growth acceleration is small even in symptomatic patients. However, as shown in our study bone age advancement can still occur. Therefore, in childhood slightly elevated androgens may have a stronger effect on bone maturation than on growth velocity.

The same effect has been observed in classic CAH children with suboptimal hormonal control and consequently slightly elevated androgens. Hargitai et al described a large cohort of CAH patients with an increased acceleration in bone maturation during the prepubertal years without an increase in growth velocity. [23] This resulted in an advance in bone age at the start of puberty, which was the main reason for the reduced peak growth velocity and reduction in adult height.

In our study group we observed a larger variation in growth acceleration below the age of 3 yrs. This is probably not a result of androgen exposure, but of target seeking, since there is a clear movement towards the 0 SD, the height as expected based on the target height.

There are some limitations to our retrospective study. The number of available growth data per patient was variable. Furthermore, because of the limited data on bone age of the individual patients we could not determine whether there is a continuous increase of bone age acceleration over time or whether the significant increase of BA_C over time is a result of differences in bone age development in symptomatic and asymptomatic patients. Further prospective studies are necessary to analyze bone age advancement over time in this patient category in more detail.

Our findings have important implications for both diagnosis and follow-up of children with NC-CAH, but also of patients with other conditions of androgen excess:

1. In patients presenting with signs of precocious pubarche in the absence of increased growth velocity, the diagnosis of NC-CAH cannot be excluded without further appropriate diagnostic studies such as bone age evaluation and biochemical analysis.
2. In asymptomatic, untreated NC-CAH patients regular measurements of growth velocity should be combined with bone age assessment at regular intervals, e.g. yearly. Treatment with glucocorticoids should be considered when signs and symptoms of androgen excess become manifest and/or when bone age advancement becomes pronounced. [1,17,18]

3. Monitoring of both classic and non classic CAH children who are treated with glucocorticoids should include evaluation of growth velocity as well as bone age determination at regular intervals to determine the effect of both subtle androgen excess and of glucocorticoid treatment.

Conclusion

In untreated NC-CAH children growth acceleration is small and generally not recognizable in their growth charts. BA_C is more pronounced. Therefore, the absence of an increase in growth velocity does not exclude the diagnosis NC-CAH. When considering the diagnosis and when monitoring NC-CAH patients over time, the emphasis should be on both BA_C as well as on growth acceleration.

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Chapter 3

Salivary morning androstenedione and 17-hydroxyprogesterone levels in childhood and puberty in patients with classic congenital adrenal hyperplasia

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Clin Chem Lab Med 2015; 53(3): 461-8

Abstract

Background Treatment of congenital adrenal hyperplasia due to 21-hydroxylase deficiency can be monitored by salivary androstenedione (A) and 17-hydroxyprogesterone (17OHP) levels. There are no objective criteria for setting relevant target values or data on changes of 17OHP and A during monitoring.

Methods We evaluated A and 17OHP levels in nearly 2000 salivary samples collected during long-term treatment of 84 pediatric patients with classic 21-hydroxylase deficiency.

Results A and 17OHP levels and its ratio 17OHP/A remained constant from 4 to 11 years with no sex-related differences. During puberty, A and 17OHP levels both increased, starting at earlier age in girls than in boys. The ratio 17OHP/A declined. Normalized A concomitant with elevated 17OHP [1.43 nmol/l (0.46 – 4.41) during prepuberty; 2.36 nmol/l (0.63 – 8.89) for boys and 1.99 nmol/l (0.32 – 6.98) for girls during puberty] could be obtained with overall median glucocorticoid doses of 11 – 15 mg/m^2/day. A levels above the upper reference limit (URL), suggesting undertreatment, coincided with 17OHP levels $\geq$10 times the URL. The percentage of A levels above the URL was 16% at ages 4 – 8 years, but increased to 31% for girls at 16 years and 46% for boys at 17 years.

Conclusions Normalized A consistent with 17OHP three times the URL during prepuberty and normalized A consistent with 4 – 6 times the URL during puberty could be obtained by moderate glucocorticoid dosages. A constant 17OHP/A ratio during prepuberty suggested absence of adrenarche. During puberty, a higher percentage of samples met the criteria for undertreatment, especially in boys.

Introduction

Congenital adrenal hyperplasia (CAH) is a group of inherited disorders caused by deficiency of one of the enzymes involved in the biosynthesis of adrenal steroids. The most frequent cause of CAH is deficiency of the enzyme 21-hydroxylase due to a mutation in the *CYP21A2* gene, which accounts for approximately 90% – 95% of the cases of CAH.

21-Hydroxylase deficiency results in impairment of the production of cortisol and, depending on the residual enzymatic activity, also of aldosterone. Due to lack of a negative feedback of cortisol to the pituitary gland, adrenocorticotrophic hormone (ACTH) concentration increases. ACTH stimulates the adrenal gland to produce cortisol. However, because of the 21-hydroxylase block, conversion of 17-hydroxyprogesterone (17OHP) to 11-deoxycortisol is impaired. 17OHP accumulates and subsequently converts into androstenedione (A) and testosterone. These androgens are responsible for virilization of female external genitalia, precocious puberty in both sexes and irregular menstruation in adolescence and adulthood. [1 – 4]

Variability in severity of the mutation results in various degrees of residual enzymatic activity of 21-hydroxylase and different clinical and biochemical presentations. [1, 3, 4] Therefore, 21-hydroxylase deficiency is divided into classic and non classic forms. The classic forms are the salt wasting (SW) and non-salt wasting, simple virilizing (SV) types. Residual enzymatic activity is 0% in SW and 1% – 2% in SV. In both classic forms there is insufficient cortisol production and excess androgen production. Additionally, aldosterone production is impaired in SW. In the non classic, late onset form of 21-hydroxylase deficiency residual enzymatic activity is 20% – 50%. Both cortisol and aldosterone production are generally sufficient, while adrenal androgen production is still slightly increased. [1, 3, 4]

The purpose of treatment of classic CAH is to overcome glucocorticoid and mineralocorticoid deficiency and suppress elevated levels of adrenal androgens. Glucocorticoid substitution results in lowering of ACTH secretion by restoring the negative feedback to the pituitary gland, thereby also lowering adrenal androgen production. Unfortunately, often supraphysiological doses of glucocorticoids are necessary to suppress adrenal androgen production with the potential risk of poor growth, hypertension and cushingoid features. In contrast, undertreatment might lead to persistently elevated androgens with premature epiphyseal maturation and loss of growth potential. Therefore, monitoring of glucocorticoid treatment is important. [2, 5 – 8]

Monitoring of glucocorticoid therapy may be accomplished by measuring early morning serum 17OHP and A concentrations. [2, 5, 6, 9] However, a venipuncture is inconvenient for children. Venipuncture causes stress, which may activate the

ACTH-adrenal axis and influence the actual levels of steroids. Saliva has been found to be an excellent alternative and collection is almost stress free. [10 – 12] Collection can be done at home before the morning glucocorticoid dose, reflecting the endogenous production of androgens and the ACTH pressure on the adrenal gland. Salivary 17OHP and A levels have been found to correlate well with serum values. [10 – 12]

There are no objective criteria for setting relevant target values for A and 17OHP in CAH patients. According to clinical guidelines A levels should be normalized, but 17OHP levels within the normal range indicate overtreatment. [5, 6] Furthermore, no data on changes of A and 17OHP levels during long-term treatment are available. Charmandari et al. [13] indicated that puberty imposes increased difficulty in attaining adrenocortical suppression despite optimal substitution therapy and adherence to medical treatment.

The objective of the present study was to retrospectively evaluate the morning levels of A and 17OHP measured in nearly 2000 salivary samples during long-term glucocorticoid treatment of a large cohort of pediatric patients with 21-hydroxylase deficiency. We determined androgen changes during childhood and puberty and estimated the extent of androgen control.

Patients and methods

Patients

The data of 84 patients with classic 21-hydroxylase deficiency, treated by paediatric endocrinologists at the Radboud University Medical Center Nijmegen in the period 1981 – 2010, were collected in a local database and analyzed anonymously. All collected data were part of the routine follow-up within patient care. The retrospective analysis was approved by the local Ethical Committee. No informed consent was asked because of the historical and anonymous evaluation of the data.

Sixty-five patients were classified as having the SW form (32 females, 33 males) and 19 patients as having the SV form (9 females, 10 males) of CAH. Classifications were based on DNA mutation analysis, clinical presentation (genital ambiguity in females, SW crisis) and/or biochemical measurements (serum/plasma ACTH, cortisol, androgens, renin and aldosterone at diagnosis).

Therapeutic follow-up within our center was started at 0.3 years of age (median; 75% of patients <4.0 years, 25% between 4.0 and 16.2 years) in SW and 4.0 years of age (median; 75% <5.2 years, 25% between 5.2 and 10.2 years) in SV patients. Some patients were referred to our clinic after initial treatment in another center. Median duration of follow-up was 12.8 years (range 3.2 – 19.0) in SW and 11.9 years (range 6.1 – 18.6) in SV patients.

Patients were treated with hydrocortisone, divided into three daily doses or with dexamethasone in one or two daily doses in some postpubertal patients. In case of mineralocorticoid requirement, fludrocortisone acetate was given. Glucocorticoid doses were related to the body surface area, expressed in milligrams per square meter. For comparability, all glucocorticoid formulations were transformed into equivalent doses of hydrocortisone using dosage equivalents based on growth retarding and androgen suppressing effects, i.e., 10 mg hydrocortisone = 0.125 mg dexamethasone. [14, 15] The surface area (SA) of the total body was calculated as: ln(SA) = −3.751 + 0.422* ln(H) + 0.515*ln(W), in which ln is the natural logarithm, SA is surface area in square meters, H is height in centimeters, and W is weight in kilograms. [16]

During follow-up, the therapy was monitored as described earlier [15] every 3 – 6 months by evaluating clinical symptoms, length and weight, bone age once a year and biochemical monitoring (see below). Patient numbers, duration of follow-up and medication are summarized in Table 1.

Biochemical monitoring

Saliva was obtained from children older than 3 years of age (for younger children salivary collection is hard to apply). Samples were collected four times a year before routine visits to the outpatient clinic of our hospital. Saliva samples were taken before administration of the glucocorticoid dose between 07.00 and 09.00 a.m. by asking the patient to salivate in a polypropylene tube (Greiner Bio-one). Clear instruction was given to prevent blood contamination in the samples. After collection, samples were sent to the laboratory and frozen at –20°C until analysis.

Three milliliters of saliva were extracted by diethyl ether and purified by paper chromatography as previously described. [10, 17, 18] A and 17OHP levels in the elutes were measured by in-house radioactive immunoassays. [10] In the immunoassays antibodies raised in sheep against androstenedione-19-carboxymethylether and against 11-deoxylcortisol-21-hemisuccinate, conjugated to BSA (ICN Biomedicals Inc.) were used. In the time period that is covered by this study (almost 30 years), this laboratory method remained unchanged. Long-term quality was assessed by control material, consisting of serum and saliva pools. The limit of detection of both A and 17OHP in saliva was 0.02 nmol/l. Intra- and inter-assay coefficients of variation were <8% for A and <9% for 17OHP.

Upper reference limits (URL) of morning A and 17OHP levels were 0.24 and 0.45 nmol/l, respectively, for children ≤11 years of age, independent of sex. [10] URL of morning A and 17OHP levels were 1.10 and 0.32 nmol/l, respectively for female adolescents/adults and 0.96 and 0.61 nmol/l, respectively for male adolescents/adults (URL was determined by logarithmic transformation of the data of previous studies [10, 19]) and subsequently, calculation of the 95% reference interval).

Data analysis

For descriptive and statistical analyses the data were categorized in intervals of years or by pubertal stage of the patients. Data collected between 3 and 19 years of age were analyzed. Data obtained between ages 3 and 4 were categorized as 4 years, between ages 4 and 5 as 5 years and so on. Pubertal stage was categorized as prepubertal or pubertal. Prepubertal stage was defined as Tanner stage <2. Pubertal stage was defined as Tanner stage ≥2. Tanner stage 2 was reached at breast stage M2 for girls and at a testicular volume ≥4 ml for boys. [20]

As the distribution of steroid concentrations was skewed in several groups, results are expressed as medians and ranges. Ranges are defined as minimal to maximal value, when outliers are excluded. Outliers are defined as values >1.5 times the interquartile range from the edge of the box in the box plot (Tukey's Hinges) as calculated from SPSS. One way Kruskal-Wallis analysis was used to analyze overall differences between groups. Mann-Whitney U-test was used as a post hoc test to consider differences between two groups. Overall, p-values <0.05 were considered statistically significant. When post hoc tests (for various age differences) were performed, a p-value <0.01 was considered significant because of repeated testing. The ratio 17OHP/A was calculated for A levels >0.05 nmol/l as very small A values may influence the ratio disproportionally. SPSS version 20 was used to perform statistical analysis.

Results

Glucocorticoid medication

Glucocorticoid doses are shown in Table 1. Median glucocorticoid doses were between 11 and 15 mg/m^2/day for the various subgroups. Glucocorticoid doses were significantly higher in SV than in SW patients in all subgroups and higher in boys than in girls (except for SW patients of 4 – 11 years). In the subgroups SW male and SV female slightly higher doses were given at 12 – 19 than at 4 – 11 years, while in the subgroup SW female lower doses were given at 12 – 19 than 4 – 11 years. The wide range of doses was due to incidental adjustment of individual daily dosages using hormonal and linear growth data.

Salivary A and 17OHP levels during glucocorticoid treatment

Figure 1 and Table 2 present the morning salivary A and 17OHP levels and the ratio 17OHP/A. In Figure 1 the levels are plotted against age and in Table 2 the data are categorized by pubertal stage.

The total number of results was 1892 for A, 1913 for 17OHP and 1462 for the ratios of 17OHP/A. The number of results categorised by age varied from 18 to 169 and by pubertal stage from 311 to 500.

Table 1 Patient numbers, follow-up and medication.

	Total CAH	Salt wasting CAH	Simple virilizing CAH
Number of patients	84	65	19
Male	43	33	10
Female	41	32	9
Start follow-up, age in years	1.2 (0.0-16.2)	0.3 (0.0-16.2)	4.0 (0.0-10.2)
Duration of follow-up, years	12.8 (3.2-19.0)	12.8 (3.2-19.0)	11.9 (6.1-18.6)
Glucocorticoid dose, mg/m^2/day			
4-11 years	12.2 (4.6-22.8)	11.7 (4.6-21.1)[b]	14.2 (4.9-23.5)
Male	12.5 (4.6-23.3)	11.9 (4.6-20.4)[b]	14.8 (6.1-24.1)
Female	11.9 (4.8-22.2)[c]	11.5 (4.8-21.7)[b]	12.8 (4.9-21.1)[c]
12-19 years	12.6 (4.2-23.0)	11.6 (4.2-22.2)[b]	14.6 (8.0-23.8)[a]
Male	13.3 (6.6-23.1)[a]	12.3 (6.6-22.2)[a,b]	14.9 (8.0-27.1)
Female	11.9 (4.2-22.9)[c]	10.9 (4.2-22.2)[a,b,c]	13.7 (8.1-21.1)[a,c]

All data are given as medians and ranges. CAH, congenital adrenal hyperplasia. Glucocorticoid dose expressed as hydrocortisone equivalents [14 – 16]. [a] Significant difference in glucocorticoid dose between 4 – 11 vs. 12– 19 years of age; p<0.05; [b] Significant difference in glucocorticoid dose between SW and SV patients; p<0.05; [c] Significant difference in glucocorticoid dose between male and female; p<0.05.

Prepuberty

Median A levels remained constant from 4 years (0.10 nmol/l, range <0.02 – 0.28) to 11 years (0.11 nmol/l, range <0.02 – 0.64). Median 17OHP levels also remained constant during the age period of 4 – 11 years (1.06 nmol/l, range <0.02 – 6.83). The median 17OHP/A ratio was about 11 (range 0.17 – 33.21) during this period.

Puberty

In the overall group, median A concentration significantly increased at the age of 13 years (0.29 nmol/l; range <0.02 – 1.21; p <0.01) when compared to the previous years. The maximal value was reached at the age of 16 years (median 0.72 nmol/l; range <0.02 – 2.71) and gradually, but not significantly, declined to 0.45 nmol/l (range 0.07 – 1.30) at 19 years of age (Figure 1).

Similar to A, 17OHP levels started to increase significantly at the age of 13 years (median 1.79 nmol/l; range <0.02 – 10.81; p <0.01). A maximum of 17OHP was reached at 15 years (median 3.40 nmol/l; range <0.02 – 11.70), remained at this level until 17 years and slightly, but not significantly, decreased at 18 and 19 years of age. The ratio 17OHP/A was 7.09 (range 0.20 – 22.67) at 12 years of age and significantly (p <0.01) decreased to 2.81 at 19 years of age (range 0.28 – 7.10).

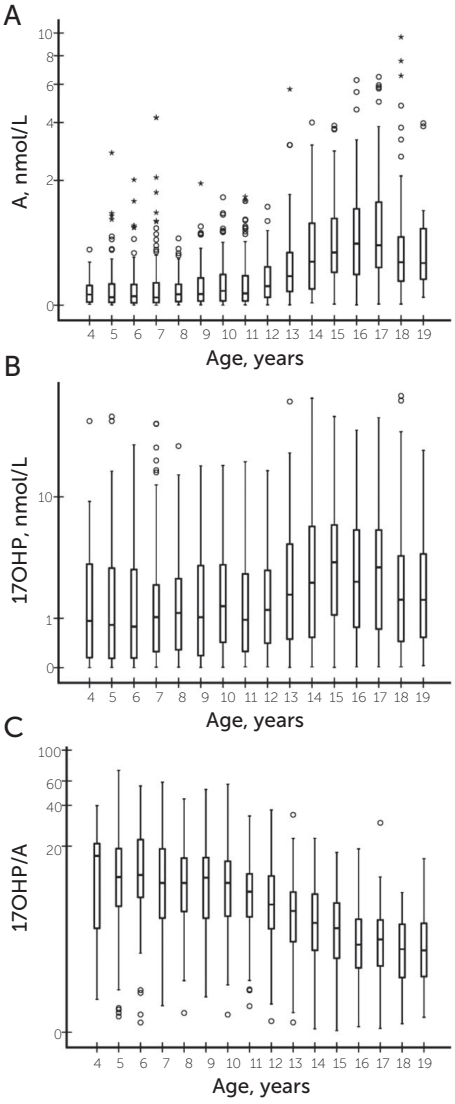

Figure 1 Morning salivary A (**A**) and 17OHP (**B**) levels (nmol/l) and the ratio 17OHP/A (**C**) of CAH patients, categorised by age in years. Patients were treated with glucocorticoids.

Results are expressed as medians and ranges (logarithmic scale). A, androstenedione; 17OHP, 17OH progesterone. Points which extend more than 1.5 times the interquartile length from the edge of the box are illustrated by 'o' and more than 3.0 times the interquartile length from the edge of the box are illustrated by '*'.

When patients were categorized by Tanner stage (Table 2), the median salivary A level was 4.7 times higher, while median 17OHP was about two times higher in pubertal compared to prepubertal children. Correspondingly, the ratio 17OHP/A was significantly lower in pubertal than in prepubertal children (p <0.05).

Table 2 Morning salivary A and 17OHP levels (nmol/l) and the ratio 17OHP/A at prepuberty (Tanner stage <2) and puberty (Tanner stage ≥2) of patients with classic 21-hydroxylase deficiency, who are treated with glucocorticoids.

	N	Median	Range
Androstenedione			
Prepuberty			
Male	500	0.09	<0.02-0.52
Female	311	0.09	<0.02-0.45
Total	811	0.09	<0.02-0.51
Puberty			
Male	465	0.41[b]	<0.02-2.12
Female	470	0.42[b]	<0.02-1.65
Total	935	0.42[b]	<0.02-1.80
17OHP			
Prepuberty			
Male	508	0.96	<0.02-6.91
Female	314	1.00	<0.02-6.12
Total	822	0.99	<0.02-6.60
Puberty			
Male	470	2.31[a.b]	<0.02-14.47
Female	473	1.72[b]	<0.02-8.89
Total	943	1.93[b]	<0.02-11.14
17OHP/androstenedione			
Prepuberty			
Male	319	10.47	0.17-31.96
Female	199	11.01	0.20-35.29
Total	518	10.81	0.17-34.12
Puberty			
Male	424	5.40[a.b]	0.03-17.18
Female	417	4.02[b]	0.03-12.73
Total	841	4.76[b]	0.03-15.00

All data are given as medians and ranges. A, androstenedione; 17OHP, 17OH progesterone. [a] Significant difference between male and female; p <0.05; [b] Significant difference between prepubertal and pubertal; p <0.05.

Gender

There were no significant differences in A concentrations between prepubertal boys and girls. A levels were twice as high in girls as in boys at 12 and 13 years, i.e., 0.29 (range <0.02 – 0.93) vs. 0.13 (range <0.02 – 0.46) nmol/l, respectively at 12 years (p <0.05) and 0.41 (range <0.02 – 1.57) vs. 0.24 (range <0.02 – 0.91) nmol/l, respectively at 13 years (p <0.05). At 14, 15 and 16 years of age, there were no significant differences between the sexes. At 17 years of age, A levels were significantly lower in girls than in boys, i.e., 0.56 (<0.02 – 1.71) vs. 0.83 (0.08 – 5.00) nmol/l, respectively (p <0.05). When patients were categorised by Tanner stage there were no significant differences in A between boys and girls.

17OHP levels were significantly higher in girls than in boys at the age of 6 years, i.e., 1.48 (<0.02 – 7.90) vs. 0.39 (<0.02 – 5.60) nmol/l, but were about two times lower in girls than in boys during the pubertal period (significant at 11, 15, 16 and 17 years; p <0.05). When categorised by pubertal stage, the difference in 17OHP levels between boys and girls was still present.

Salivary androgen levels suggesting optimal versus undertreatment

In Table 3 the salivary data are divided in three subgroups, i.e., 1) A below URL and 17OHP above URL suggesting optimal treatment; 2) A and 17OHP below URL suggesting overtreatment; 3) A and 17OHP above URL suggesting undertreatment. In the group representing optimal treatment median A levels were 0.4 –0.5 times the URL, i.e., 0.11 (<0.02 – 0.24) nmol/l for children of 4 – 11 years of age, 0.37 (0.02 – 0.96) nmol/l for boys and 0.50 (<0.02 – 1.10) nmol/l for girls of 12 – 19 years of age. Median 17OHP was 1.43 (0.46 – 4.41) nmol/l for the age group 4 – 11 years, which corresponds with three times the URL. Median 17OHP was 2.36 (0.63 – 8.89) nmol/l for boys and 1.99 (0.32 – 6.98) nmol/l for girls of 12 – 19 years of age, corresponding with four and six times URL, respectively.

A and 17OHP levels suggesting undertreatment were 2 and 10 times URL, respectively for the age group 4 – 11 years. For the age group 12 – 19 years median A levels were also up to 2 times URL, while 17OHP levels were 16 – 18 times URL for girls and boys. When categorized by age, about 16% of the androgen concentrations met the criteria for undertreatment at 4 – 8 years of age, which substantially increased to 31% for girls at 16 years and 46% for boys at 17 years of age.

Table 3 Morning salivary A and 17OHP levels (nmol/l) in patients with classic 21-hydroxylase deficiency, divided in 3 subgroups: 1) A below URL and 17OHP above URL suggesting optimal treatment; 2) A and 17OHP below URL suggesting overtreatment; 3) A and 17OHP above URL suggesting undertreatment.

	N	Androstenedione	17OHP
4-11 years			
Total	1024	0.10 (<0.02-0.55)	1.07 (<0.02-6.83)
1. Optimal	429	0.11 (<0.02-0.24)	1.43 (0.46-4.41)
2. Overtreated	342	0.02 (<0.02-0.09)	0.12 (<0.02-0.45)
3. Undertreated	252	0.40 (0.24-1.02)	4.70 (0.49-14.50)
12-19 years			
Total	868	0.44 (<0.02-1.97)	2.02 (<0.02-11.51)
Male	485	0.41 (<0.02-2.20)	2.32 (<0.02-15.04)[a]
Female	383	0.47 (<0.02-1.71)	1.78 (<0.02-8.50)
1. Optimal	464	0.45 (<0.02-1.10)	2.21 (0.32-7.70)
Male	238	0.37 (0.02-0.96)[a]	2.36 (0.63-8.89)[a]
Female	226	0.50 (<0.02-1.10)	1.99 (0.32-6.98)
2. Overtreated	211	0.10 (<0.02-0.45)	0.15 (<0.02-0.60)
Male	121	0.10 (<0.02-0.41)	0.24 (<0.02-0.60)[a]
Female	90	0.10 (<0.02-0.52)	0.07 (<0.02-0.38)
3. Undertreated	193	1.53 (0.96-3.73)	7.64 (1.48-25.00)
Male	126	1.60 (0.96-4.60)	9.52 (1.55-27.11)[a]
Female	67	1.47 (1.11-2.32)	5.69 (1.48-17.00)

Results are expressed as medians and ranges. A, androstenedione; 17OHP, 17OH progesterone; URL, upper reference limit. [a] Significant difference between male and female; $p < 0.05$.

Discussion

We retrospectively evaluated morning salivary A and 17OHP concentrations in nearly 2000 samples of a large pediatric cohort of classic CAH patients (21-hydroxylase deficiency). The patients were treated with glucocorticoids and monitored in our outpatient clinic from childhood to adulthood. We described changes of salivary A and 17OHP during childhood and puberty. In addition, we evaluated the levels in light of optimal treatment and undertreatment by a combination of A and 17OHP. To the best of our knowledge no published data on salivary A and 17OHP levels during long-term treatment of CAH patients are

available. The present analysis is based on the same, accurate and validated steroid hormone assays throughout the observation period of about 30 years. This method employs preliminary solvent extraction and chromatography prior to the immunoassay, preventing interference from cross reacting steroids. [10, 17, 18]

Moderate median glucocorticoid dosages of 11 – 15 mg/m^2/day were used, which is in line with current international guidelines. [5, 6] Our data show that slightly higher dosages of hydrocortisone were needed in boys than in girls and in SV than in SW patients to suppress adrenal androgens. Probably, in these patients higher hydrocortisone dosages were necessary to normalize the chronically activated pituitary adrenal axis. Bonfig et al. [21] advised not to exceed the hydrocortisone dosage above 17 mg/m^2/day during puberty as supraphysiological dosages of glucocorticoids may have adverse effects on pubertal growth and final height. The balance between overtreatment and undertreatment is still a clinical challenge.

During prepuberty A and 17OHP levels remained constant with no sex-related differences. In healthy children adrenarche starts around 6 years of age, initiated by an increase in CYP17 activity within the adrenal cortex. Especially the 17,20-lyase component of the CYP17 enzyme is enhanced due to increase in cytochrome B5 activity that serves as a co-factor for 17,20-lyase. Consequently, adrenal androgen production [DHEA(S), and A] increases with a decrease in 17OHP/A ratio during childhood. Our data showed no change of the 17OHP/A ratio, suggesting no increase in CYP17 activity and therefore absence of clear adrenarche in CAH patients, corresponding to other studies. [22, 23] Chronic hydrocortisone treatment may suppress the zona reticularis activation with consequently absence of adrenarche.

Significant increases of salivary A and 17OHP levels were observed during puberty indicating gonadal activity and steroid production within the testes or ovaries. The pattern of A and 17OHP changes in CAH patients should be compared with a reference population of healthy children, categorised by sex and age or Tanner stage. Our reference values were not detailed in this respect. In the literature few reports are available on serum and even less on saliva reference values. [9, 24 – 26] Additionally, there is a lack of international standardisation of A and 17OHP assays, cross-reactivity in immunoassays causing overestimation, and use of different techniques (i.e., immunoassay vs. LC-MS/MS), complicating comparison of studies.

Kushnir et al. [25] studied morning serum A levels in more than 2500 healthy children. Concentrations of A increased during puberty reaching a maximum at the age of 20 – 30 years and gradually declining afterwards. In smaller studies (n = 132 – 337) comparable changes of A and 17OHP in serum or saliva were found during puberty versus childhood. [9, 24, 26] Kushnir et al. [25] observed that serum A levels reached adult values at Tanner stage 3 for girls and at Tanner stage 4 – 5 for boys. Our results in CAH patients correspond with the findings of Kushnir et al. [25] that A levels increase sooner in girls reflecting earlier gonadal maturation. [27] This

suggests that reference values of A and 17OHP based on Tanner stage rather than age should be used for the monitoring of glucocorticoid therapy in CAH patients.

In the literature, serum A levels are about 2 – 3 times higher in healthy females than in males during the age period of 12 – 19 years. [9, 25, 26] In our CAH patients no sex-related differences in A levels were visible when categorized by pubertal stage. This may be explained by the contribution of elevated A levels representing undertreatment in the overall group. Accordingly, we found gender-related differences in the levels of 17OHP during puberty with higher 17OHP in boys than in girls, whereas no gender-related differences of 17OHP were described in healthy pubertal subjects. [9, 24 – 26] When only the subgroup with levels suggesting optimal treatment was considered, A levels were higher in girls than in boys during puberty corresponding to the literature. [9, 25, 26] The sex-related difference of 17OHP levels, though, was still present.

According to clinical guidelines A levels should be normalized by treatment, but 17OHP levels within the normal range indicate overtreatment. [5, 6] We found normalized A levels associated with 17OHP levels three times URL during prepuberty and 4 – 6 times URL during puberty. A levels suggesting undertreatment were up to two times URL in combination with 17OHP levels ≥10 times URL during prepuberty and puberty. The percentage of A levels representing undertreatment was 16% during the age interval of 4 – 8 years and increased to 31% for girls at 16 years and 46% for boys at 17 years of age, suggesting less adrenal suppression during puberty. [13, 27] Several studies indicated that the cortisol clearance rate increases during puberty due to either inhibition of the activity of 11β hydroxysteroid dehydrogenase type 1 or 5α-reductase activity. [13, 27, 28] In CAH patients these changes may result in decrease of cortisol concentrations, subsequently inadequate suppression of the adrenal gland and increased production of 17OHP and A. Lack of compliance may further contribute to increases in adrenal androgen levels. During puberty, adherence to medical therapy is more difficult due to psychosocial factors. Hirsutism and acne may be important drives for girls to better adhere to medical therapy than boys. Moreover, the presence of testicular adrenal rest tumours in boys may contribute to enhanced production of 17OHP. [29] Further studies are necessary to investigate this hypothesis.

It is important to note that in the clinical laboratory liquid chromatography-tandem mass spectrometric methods (LC-MS/MS) are replacing immunoassays for steroid analysis. Various LC-MS/MS methods on serum A and 17OHP are described [9, 25, 26, 30], but only very few reports on salivary A or 17OHP are available. [31, 32] High sensitivity is needed as concentrations in saliva are low. Future research is needed to extrapolate our results to LC-MS/MS methods.

In summary, the ratio 17OHP/A remained constant during prepuberty suggesting the absence of adrenarche. During puberty, A and 17OHP levels increased at an

earlier age in girls than in boys, suggesting that reference values of A and 17OHP based on Tanner stage rather than age should be used for the monitoring of glucocorticoid therapy in pediatric CAH patients. Normalized A levels in combination with 17OHP three times URL during prepuberty and normalized A levels in combination with 17OHP 4 – 6 times URL during puberty could be obtained with moderate glucocorticoid dosages of 11 – 15 mg/m^2/day. A levels suggesting undertreatment were up to two times URL, while 17OHP levels were ≥10 times URL during prepuberty and puberty. During puberty the highest percentage of androgen levels consistent with undertreatment was found in boys of 17 years of age.

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Chapter 4

Long term follow-up of children with classic congenital adrenal hyperplasia: time for more age-specific treatment strategies?

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Abstract

Background In congenital adrenal hyperplasia (CAH), achieving the balance between overtreatment and undertreatment remains challenging. Final height (FH) serves as a long term outcome measure. We aimed to identify age-specific factors that contribute to FH.

Methods We retrospectively evaluated longitudinal data of 39 pediatric CAH patients. We analyzed height and bone age at diagnosis or 4 years of age, at the start of puberty, and at FH. Height data were corrected for parental height and secular trend. Hydrocortisone use and salivary steroid levels were studied longitudinally throughout childhood and puberty.

Results Median FHSDS corrected for target height SDS (THSDS) was -1.63. In simple virilizing CAH, median height SDS corrected for THSDS (HSDS-THSDS) decreased from -0.1 SD (females) and +2.7 SD (males) at diagnosis to -1.93 SD (females) and -1.92 SD (males) at FH. In salt-wasting CAH, HSDS-THSDS decreased from -0.04 SD (females) and -0.78 SD (males) at 4 years of age to -0.79 SD (females) and -1.27 SD (males) at FH. However, when height was corrected for bone age, no height loss occurred from diagnosis or age 4 years to FH in any of the subgroups. Hydrocortisone dose and FH were negatively associated throughout childhood and puberty, as well as androstenedione levels and FH in childhood, but not in puberty.

Conclusions In CAH, loss of growth potential already occurs in early childhood. In prepubertal children, exposure to elevated androgens is associated with decreased FH. In puberty, the growth suppressing effects of HC outweigh the negative effects of elevated androgens. Therefore, we suggest different treatment approaches in prepubertal and pubertal patients.

Introduction

Congenital adrenal hyperplasia (CAH) is caused by a deficiency of one of the enzymes involved in adrenal steroid synthesis, in most cases 21-hydroxylase. This results in impaired synthesis of cortisol. Due to reduced negative feedback to the hypothalamus and pituitary gland, the pituitary secretion of ACTH is increased. Consequently, steroid hormone precursors prior to the enzymatic block accumulate and are shunted into the androgen-synthesis pathway. [1] CAH represents a continuum of disease, depending on the severity of the enzyme deficiency. The severe, classic form can be subdivided into a salt wasting (SW) and a simple virilising (SV) subtype, both with severe cortisol deficiency. Patients with SW-CAH have a residual enzyme activity of <1% leading to additional aldosterone deficiency. A residual enzyme activity of 1-2% ensures sufficient aldosterone production to prevent severe salt loss, and results in SV-CAH. The non classic form (NC-CAH) is milder, with a residual enzyme activity of 20-50%. In NC-CAH, basal cortisol and aldosterone levels are usually normal. Androgens are still slightly elevated which may lead to clinical signs, such as premature pubarche, hirsutism and menstrual disturbances. [1]

The treatment of classic CAH consists of lifelong use of glucocorticoids and if necessary also mineralocorticoids to prevent adrenal and salt wasting crises. The glucocorticoids suppress the secretion of adrenal androgens due to decreased ACTH release. However, supraphysiological doses are usually necessary to suppress ACTH and consequently androgen production. [2] Since both over- and undertreatment may influence patient outcomes negatively, glucocorticoid treatment in CAH is a balancing act. Undertreatment results in chronically elevated adrenal androgens leading to premature epiphyseal maturation and a reduced final height. [3] Overtreatment may result in cushingoid features, hypertension, and osteoporosis. It may also reduce final height due to a reduction of growth hormone secretion and action, by a disturbance of calcium metabolism and by direct effects on the growth plate. [2, 4, 5] In clinical practice, both over- and undertreatment are common. [6] Despite the increasing understanding of pathophysiological mechanisms and the development of guidelines for clinical practice, [2] questions remain on how to optimize the care of CAH children, and monitoring and treatment strategies differ between treatment centers. [2, 4]

Careful monitoring of treatment is an important aspect of the care of children with 21-hydroxylase deficient CAH. The levels of the adrenal steroid precursors 17-hydroxyprogesterone (17OHP) and androstenedione (A) are used as markers for long term follow-up. These precursors can be measured in blood, urine, saliva, or dried filter paper blood samples. [4] In our center, we introduced salivary 17OHP

and A measurements as a routine monitoring tool for CAH patients more than 30 years ago. Saliva collection is non-invasive and less stressful than taking blood samples, especially in pediatric patients. Samples can be collected at home several times per day reflecting the diurnal rhythm of steroid levels. Previous research showed that salivary levels of 17OHP and A reflect plasma steroid levels reliably. [7-9]

In this study, we retrospectively evaluated the monitoring and treatment strategy in our pediatric CAH population over the past 30 years. We evaluated longitudinal data from diagnosis to final height regarding corticosteroid use, salivary steroid levels and growth. We aimed to determine age-specific characteristics and define treatment recommendations, to improve long term clinical outcome.

Patients and methods

Patients

For this retrospective evaluation, we used the longitudinal Radboudumc CAH database. Longitudinal data have been collected for all CAH patients treated at the department of pediatric endocrinology of the Radboud university medical center, Nijmegen, the Netherlands, born from 1957 onwards (216 patients in total). The database contains data on mutation analysis and parental height, longitudinal data on height, weight, Tanner stages and doses of glucocorticoid and mineralocorticoid medication (nearly 6000 visits to the outpatient clinic), bone age (approximately 1500 occasions), and salivary steroid profiles (17OHP and A) at nearly 4000 occasions.

Patients were included in this study if they were diagnosed with classic CAH due to 21-hydroxylase deficiency, were born between 1980 and 1997, and were treated in our hospital from early childhood onwards with at least annual visits to our outpatient clinic. We limited the inclusion to patients born between 1980 and 1997 because salivary steroid measurements were introduced in the early 1980's and the method for analysis did not change during this time period.

In this historical cohort, the patients were not diagnosed by neonatal screening since the Dutch CAH screening program was not implemented until the year 2002. The diagnosis of SW-CAH or SV-CAH was based on clinical and biochemical data and confirmed by mutation analysis. The *CYP21A2* mutations were categorized into different mutation groups based on *in vitro* 21-hydroxylase activity: groups null (0%), A (0-1%), B (1-5%), and C (20-50%). [10] Mutations in groups null and A are associated with SW-CAH, group B with SV-CAH, and group C with NC-CAH. The phenotype is usually determined by the least severe mutation. In case of discrepancy between phenotype and genotype, patients were classified according to their phenotype.

Patients with NC-CAH were excluded because of the variation in treatment strategies in these patients. Patients with co-morbidity or using additional medication that might influence final height were also excluded. No informed consent was obtained because of the retrospective analysis of de-personalized data.

The Radboudumc pediatric CAH treatment and monitoring protocol

All patients are treated with hydrocortisone (HC), generally below 15 mg/m^2/day, divided in three daily doses with the highest dose in the evening. The patients visit the outpatient clinic every 3-4 months. Before each visit, patients collect saliva samples at home, three times during one day before taking their HC. The saliva is purified by paper chromatography and 17OHP and A are measured by in-house radioactive immunoassays as previously described. [11] Based on the results we adjust the HC dose, with the aim to decrease the A levels to within age- and sex-appropriate reference ranges without completely suppressing 17OHP levels to avoid overtreatment. [2] During each visit, height, weight, and blood pressure are recorded. Bone age (BA) assessment is performed yearly until final height is reached.

Data collection and definition

From the Radboudumc CAH database the following data were collected:

- Gender
- Mutation analysis and clinical data
- Age, height, and BA at diagnosis in SV-CAH patients, at the age of 4 years in SW-CAH patients, and at the start of puberty in all patients. The start of puberty was defined as Tanner stage M2 in girls and a testicular volume of $\geq$ 4 ml in boys. Height SDS (HSDS) was calculated using the 1997 Dutch growth study reference values. [12] Furthermore, HSDS was corrected for BA. BA was corrected for calendar age (BA-CA).
- Final height (FH), defined by less than 0.5 cm growth in the previous 12 months or by a BA >17 years. Final height SDS (FHSDS) was calculated using the 1997 growth study reference values. The formulas used were FHSDS = (final height in cm – 184)/7.1 for males, and FHSDS = (final height in cm – 170.6)/6.5 for females. [12]
- Parental height, used to calculate target height (TH) with the 1997 growth study formulas, which also account for secular trend. For males: TH in cm = (paternal height in cm + maternal height in cm + 13)/2 + 4.5. For females: TH in cm = (paternal height in cm + maternal height in cm - 13)/2 + 4.5. [12] Target height SDS (THSDS) was calculated using the same formulas as used for FHSDS. In the analyses, all height data were corrected for target height: HSDS-THSDS and FHSDS-THSDS.
- Weight at FH. Body mass index (BMI) was calculated as weight(kg)/height(m)^2.

- Glucocorticoid doses. HC doses were recorded from the age of four years until the patient reached FH. They were expressed as mg/m^2/day. Mineralocorticoid use was not recorded because of the expected limited effects on our treatment outcome measures.
- Salivary steroid measurements. All morning measurements of 17OHP and A were recorded from the age of four years until the patient reached FH. For SV-CAH patients, the salivary steroid measurements in the first three months after diagnosis were not used for analysis since the levels in this stage are more likely to represent disease activity than treatment effect. SD scores for 17OHP (17OHP SDS) and A (A SDS) levels were calculated for each patient to reflect variability in hormonal control.

Statistical analysis

Descriptive analyses were performed to check the age and growth data for normal distributions. Because of the small numbers in the subgroups by gender and CAH subtype, all of these data were presented as medians plus ranges. Kruskal-Wallis and Mann-Whitney U tests were used to test differences among the groups, with p-values <0.05 being considered statistically significant. To take full advantage of the continuously recorded HC doses and the many salivary steroid measurements, while accounting for differences in the numbers of measurements among patients, we performed linear mixed model analyses to estimate the mean daily HC doses and the mean steroid levels with 95% confidence intervals (CI) for all patients, as well as separately by gender, subtype, and time period (childhood vs. puberty). Linear regression analyses, weighted for the square root of the number of measurements, were used to assess the effects of individual mean HC dose and steroid levels as well as their variability on final height (FHDSD-THSDS). If needed, the regression models were adjusted for the covariables HC dose, gender, CAH subtype, age at start puberty, and triptorelin use. All statistical analyses were performed using SPSS for Windows version 22 (IBM SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

The data of 77 CAH patients born between 1980 and 1997 were recorded in the Radboudumc CAH database. 8 patients were excluded because of a different enzymatic defect than 21-hydroxylase (11β hydroxylase- (n=5) and 3β hydroxysteroid dehydrogenase deficiency (n=3)) and 17 patients because of NC-CAH. Of the 52 patients with classic 21-hydroxylase deficiency, 2 patients were excluded because of relevant co-morbidity (severe spasticity and anorexia nervosa) and 10 patients

because of limited data and possible different treatment strategies in the first years of life, as they were diagnosed and treated elsewhere up to 8 years of age or older. In one additional patient, there was a lack of salivary steroid measurements due to non-compliance. This resulted in 39 CAH patients being eligible for inclusion in our study: 18 female and 21 male patients. 25 patients were classified as SW (12 female, 13 male) and 14 patients were classified as SV (6 female, 8 male) based on their genotype and phenotype (Supplemental Table 1). In one patient, marker analysis was performed prenatally and DNA analysis was not performed after birth. The clinical picture in this patient was consistent with SW-CAH. In 3 patients, genotype and phenotype were not consistent. They were classified according to their phenotype (2 SV, 1 SW).

Age, height, and bone age at diagnosis and at the age of 4 years

Of the 25 SW-CAH patients, 24 were diagnosed neonatally because of ambiguous genitalia and/or salt wasting crises. One boy with SW-CAH was diagnosed at the age of two years. Neonatally he was diagnosed with posterior urethral valves and his salt wasting was initially attributed to secondary pseudohypoaldosteronism. The clinical course in this patient was described in more detail elsewhere. [13] In the SW-CAH patients, at the age of 4 years the HSDS-THSDS was -0.04 in girls (range -2.82 – 0.94) and -0.78 in boys (range -1.59 – 2.03) (Figure 1a, Table 1). However, HSDS corrected for BA and THSDS was -1.30 in girls (range -4.07 – 1.70) and -1.01 in boys (range -3.39 – 1.82) (Figure 1b, Table 1).

Female SV-CAH patients were diagnosed earlier than male SV-CAH patients, at a median age of 2.3 years (range 0-4.0 years) compared to a median of 4.4 years (range 2.5-6.3 years). Bone age advancement was more pronounced in SV-CAH boys (+5.8 years). Median HSDS-THSDS at diagnosis in SV-CAH patients was -0.08 in girls (range -1.41 – +3.30) and +2.69 in boys (range +1.88 – +4.90) (Figure 1a, Table 1). However, HSDS corrected for BA and THSDS was -1.97 (range -3.93 - -1.05) in females and -3.52 (range -4.56 - -1.63) in males (Figure 1b, Table 1).

Age, height, and bone age at start of puberty

Puberty started at a median age of 9.6 years (range 7.8-13.2 years) in females and 11.3 years (range 7.3-13.8 years) in males. Eleven patients (28%) were treated with triptorelin to delay puberty in an attempt to improve final height. The median HSDS-THSDS at start of puberty showed clear differences between boys and girls ($p=0.01$), but not between CAH subtypes ($p=0.63$) (Figure 1a, Table 1). Median BA-CA was most advanced in male SV-CAH patients (+2.6 years). Median HSDS corrected for BA and THSDS was less favorable for SV-CAH patients compared to SW-CAH patients, although the HSDS for the SV patients improved compared to those at diagnosis (Figure 1b, Table 1).

Table 1 Height and bone age (BA) at diagnosis (in SV-CAH patients) or at 4 years of age (in SW-CAH patients), at Tanner stage 2, and at age of final height (FH).

	HSDS-THSDS at diagnosis / 4 yrs	HSDS corrected for BA – THSDS at diagnosis / 4 yrs	BA-CA at diagnosis / 4 yrs (years)	HSDS-THSDS at Tanner stage 2	HSDS corrected for BA – THSDS at Tanner stage 2	BA-CA at Tanner stage 2 (years)	FHSDS-THSDS
SW female (n=12)	-0.04 (-2.82 – 0.94)	-1.30 (-4.07 – 1.70) (n=10)	+0.6 (-2.4 – +2.1) (n=10)	-0.65 (-1.75 - 1.19)	-0.92 (-3.51 – 0.61)	+0.6 (-2.0 – +3.5)	-0.79 (-2.46 - -0.21)
SW male (n=13)	-0.78 (-1.59 – 2.03)	-1.01 (-3.39 – 1.82) (n=12)	+0.2 (-1.2 - +3.7) (n=12)	-0.32 (-0.85 – 1.44)	-1.14 (-2.23 – 0.46) (n=11)	+1.5 (+0.1 – +2.8) (n=11)	-1.27 (-2.60 - -0.75)
SV female (n=6)	-0.08 (-1.41 – 3.30)	-1.97 (-3.09 – -1.05) (n=5)	+2.0 (+1.3 – +3.8) (n=5)	-1.25 (-2.99 - 1.30)	-1.60 (-2.16 – 0.13)	+0.4 (-1.5 – +2.1)	-1.93 (-2.85 - -1.00)
SV male (n=8)	+2.69 (+1.88 – 4.90)	-3.52 (-4.56 – -1.63)	+5.8 (+3.6 – +7.5)	0.67 (-0.87 - 2.14)	-1.92 (-2.78 – -0.13)	+2.6 (-0.4 – +4.8)	-1.92 (-2.75 - -0.63)

SV = simple virilizing. SW = salt wasting. Height at diagnosis or at 4 years of age, at Tanner stage 2, and final height are expressed as height SD scores (HSDS) for calendar age (CA) and for BA, corrected for target height SDS (THSDS). Bone age advancement (BA-CA) is expressed as bone age corrected for calendar age in years. All data are expressed as median and range.

Final height

Median FHSDS-THSDS was -1.63 (range -2.85 - -0.21) for the entire cohort, with clear differences between the CAH subtypes (p=0.01) (Figure 1, Table 1). The female SW-CAH patients reached a FHSDS-THSDS closest to their genetic potential, median -0.79 SDS, while the median FHSDS-THSDS in male SW-CAH patients was -1.27 SDS. The median FHSDS-THSDS was almost -2 SDS in both male and female SV patients.

BMI

The median BMI at FH was 21.5 kg/m^2 (range 18.7-31.6 kg/m^2). No differences were observed between males and females and between subtypes. Associations between BMI and FH, HC dose, 17OHP level, and A level were not observed (data not shown).

Hydrocortisone use

The median number of HC doses recorded per patient was 39 with a range of 18-51. From the mixed model analysis, the mean daily HC dose for the entire group of patients was estimated to be 12.7 mg/m^2/day (95% CI 11.7-13.7) (Table 2). SV-CAH patients were treated with higher doses of hydrocortisone than SW-CAH patients (14.8 vs. 11.5 mg/m^2/day; p=0.001). No clear differences were seen between male and female CAH patients (13.3 vs. 12.0 mg/m^2/day; p=0.21) or between doses received in childhood and puberty (12.7 vs. 12.9 mg/m^2/day; p=0.18).

Salivary steroid measurements

For both 17OHP and A, a median of 31 (range 14-50) measurements were available per patient. The mean salivary 17OHP levels were lower in childhood than in puberty (p=0.000) without clear differences between males and females (p=0.20) and between CAH subtypes (p=0.11). The levels of 17OHP varied between 3 and 13 times the upper limit of the age- and gender-appropriate reference ranges (Table 2). The salivary A levels were also lower in childhood than in puberty (p=0.000), and did not differ between gender and subtypes. The mean A levels were within age- and gender-appropriate reference ranges, except for male pubertal patients. In these patients, the A levels were slightly above the upper limit of the reference ranges.

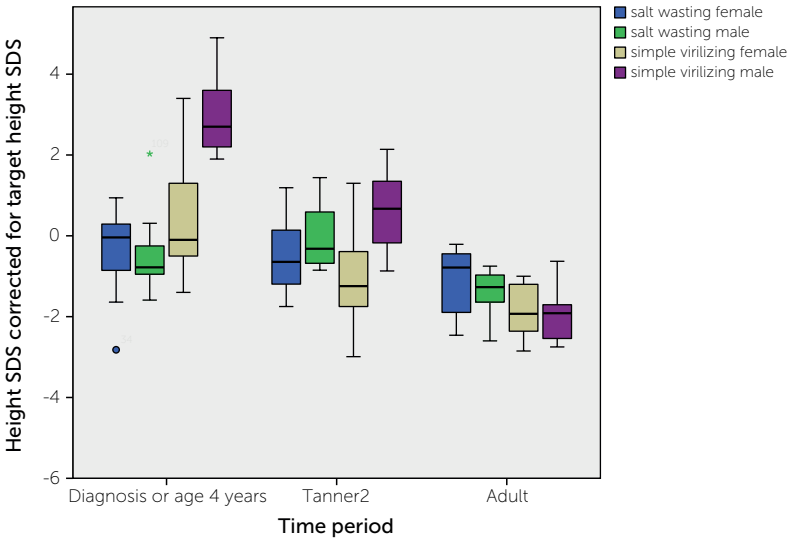

Figure 1a Height SDS corrected for target height SDS at diagnosis (SV-CAH) or at 4 years of age (SW-CAH), at Tanner stage 2, and at final height.

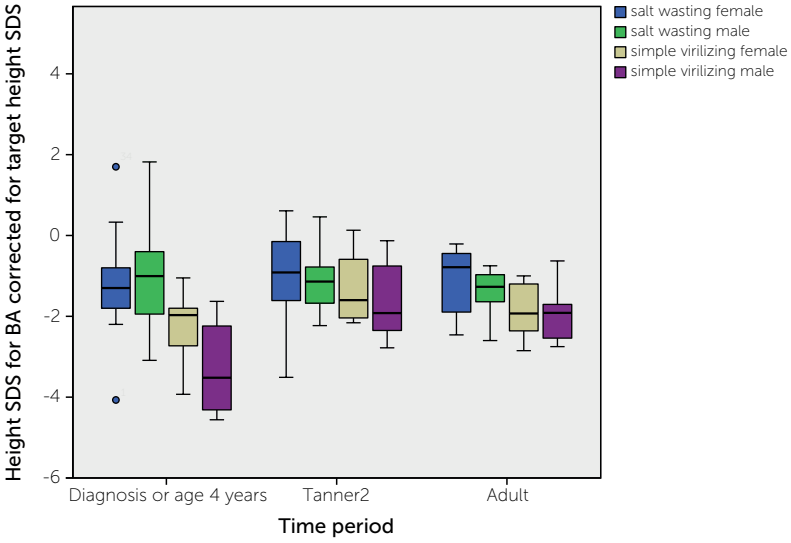

Figure 1b Height SDS corrected for bone age (BA) and target height SDS at diagnosis (SV-CAH) or at 4 years of age (SW-CAH), at Tanner stage 2 and at final height.

Table 2 Daily hydrocortisone doses and salivary steroid levels.

	All patients (n=39)	SW female childhood (n=12)	SW female puberty (n=12)	SW male childhood (n=13)	SW male puberty (n=13)	SV female childhood (n=6)	SV female puberty (n=6)	SV male childhood (n=8)	SV male puberty (n=8)
Hydrocortisone* (mean, mg/m2/day)	12.7	11.2	11.4	11.6	12.2	13.9	13.4	15.8	15.9
95% CI	11.7 – 13.7	9.8 – 12.6	9.2 – 13.5	10.6 – 12.7	11.0 – 13.4	9.6 – 18.2	11.4 – 15.5	12.4 – 19.1	12.2 – 19.5
17OHP** (mean, nmol/l)	3.23	1.44	3.99	2.20	5.09	2.47	4.46	2.59	6.71
95% CI	2.72 – 3.74	1.02 – 1.85	1.79 – 6.18	1.63 – 2.78	3.22 – 6.97	1.63 – 3.32	1.46 – 7.46	1.20 – 3.98	3.78– 9.64
Age-appropriate reference values 17OHP		0.005- 0.45	0.02 – 0.32	< 0.26	0.03 – 0.61	0.005 – 0.45	0.02 – 0.32	< 0.26	0.03 – 0.61
Androstenedione*** (mean, nmol/l)	0.46	0.18	0.75	0.16	1.02	0.15	0.57	0.21	1.16
95% CI	0.36 – 0.55	0.11 – 0.25	0.41 – 1.09	0.11 – 0.22	0.28 – 1.76	0.06 – 0.24	0.47 – 0.67	0.10 – 0.32	0.52 – 1.79
Age- appropriate reference values Androstenedione		0.03 – 0.18	0.18 – 1.10	0.04 – 0.24	0.11 – 0.96	0.03 – 0.18	0.18 – 1.10	0.04 – 0.24	0.11 – 0.96

SW = salt wasting; SV = simple virilizing. *In the mixed model for hydrocortisone, the p-values for the covariables were: p=0.18 for childhood vs. puberty, p=0.21 for gender, and p=0.001 for CAH subtype. **In the mixed model for 17OHP, the p-values for the covariables were: p=0.00 for childhood vs puberty, p=0.20 for gender, and p=0.11 for CAH subtype. *** In the mixed model for androstenedione, the p-values for the covariables were: p=0.00 for childhood vs puberty, p=0.37 for gender, and p=0.68 for CAH subtype.

Associations between hydrocortisone doses, salivary steroid levels, and final height

Both in childhood and in puberty, each additional mg/m^2/day of HC (averaged over the time period) was associated with a reduction in final height of -0.13 SDS (Table 3). Other variables included in the statistical model (gender, CAH subtype, age at start puberty and triptorelin use) did not influence the results (data not shown).

Small but statistically significant negative associations were seen between daily HC doses and salivary 17OHP and A levels in childhood (-0.16, p=0.00 and -0.01, p=0.00, respectively), whereas these associations were positive in puberty (for 17OHP +0.24, p=0.01 and for A +0.03, p=0.05).

In childhood, negative associations were observed between both 17OHP levels and 17OHP variability (17OHP SDS) and FHSDS-THSDS (-0.30, p=0.01 and -0.16, p=0.05). However, when HC dose was included in the statistical model, the association disappeared and only the associations with HC dose were statistically significant (Table 3). In puberty, the associations between FHSDS-THSDS and 17OHP levels and 17OHP variability were less strong and also disappeared completely when the results were adjusted for HC dose. For A, strong negative associations with FHSDS-THSDS were seen in childhood for both A levels and A variability, which were only slightly reduced by adjustment for HC dose, CAH subtype, and age at start of puberty. The initial slight associations between FHSDS-THSDS and A levels and A variability in puberty disappeared after adjustment for covariables.

Table 3 Associations of daily hydrocortisone dose (HC) and salivary steroid levels (17OHP and androstenedione, A) and the amount of variability in these levels (17OHP SDS and A SDS) with final height (FHSDS-THSDS).

	FHSDS-THSDS	p
Childhood		
HC dose	- 0.13	0.00
17OHP level	-0.30	0.01
Adjusted model:		
17OHP level	-0.14	0.22
HC dose	-0.11	0.01
17OHP SDS	-0.16	0.05
Adjusted model:		
17OHP SDS	-0.04	0.57
HC dose	-0.12	0.00
A level	-3.58	0.00
Adjusted model:		
A level	-2.88 *	0.03
HC dose	-0.07	0.12
A SDS	-3.75	0.01
Adjusted model:		
A SDS	-2.99 *	0.01
HC dose	-0.07	0.12
Puberty		
HC dose	- 0.13	0.00
17OHP level	-0.08	0.05
Adjusted model:		
17OHP level	-0.03	0.42
HC dose	-0.10	0.01
17OHP SDS	-0.07	0.08
Adjusted model:		
17OHP SDS	-0.02	0.66
HC dose	-0.011	0.01
A level	-0.27	0.07
Adjusted model:		
A level	-0.17 *	0.24
HC dose	-0.09	0.04
A SDS	-0.46	0.06
Adjusted model:		
A SDS	-0.23 *	0.33
HC dose	-0.09	0.04

All results represent the amount of change in final height (FHSDS-THSDS) associated with one mg/m^2/day increase in HC dose or one unit change in steroid level (averaged over the time period). The adjusted models always included both the steroid levels and hydrocortisone (HC) dose, while the adjusted models for A also included: * CAH subtype and age at start puberty.

Discussion

This study describes longitudinal data on glucocorticoid use, hormonal control, and height outcome in a unique single centre cohort of uniformly treated pediatric classic CAH patients regularly monitored by salivary steroid measurements. Although several studies previously reported reduced final height in CAH patients, [14-18] we report final height data corrected for both target height and secular trend in a homogenously treated cohort of CAH patients. When secular trend is not accounted for, height outcomes will be overestimated. Therefore, we consider our results to be a reliable representation of actual final height outcomes. Furthermore, we were able to define specific patterns related to age and CAH subtype. We showed that evaluating growth by using height measurement only is insufficient: by correction of the height for bone age as an additional method to describe growth and maturation, we unmasked a striking loss of growth potential in early childhood not visible in the regular growth charts. During puberty, no further loss of HSDS occurred.

As described in previous studies, FH was more reduced in SV-CAH patients compared to SW-CAH patients. [14, 16] This is most likely due to delayed diagnosis and treatment, resulting in more prolonged exposure to elevated androgen levels and consequently an advanced BA. The neonatal screening program that has been implemented widely, primarily to reduce salt wasting crises in SW-CAH patients, also detects SV-CAH patients. This may be beneficial for the long term height outcome in SV-CAH by shortening the period of elevated androgen exposure. [16] In SV-CAH patients, higher HC doses were needed to adequately suppress the androgen overproduction compared to SW-CAH patients, which also contributed to the more reduced FH. In the SW-CAH patients in our study however, FH was still slightly reduced despite early diagnosis and moderate HC doses. Current guidelines recommend HC doses between 10 and 17 mg/m^2/day in growing CAH patients. [2, 4] Our results show that even in these moderate supraphysiological doses a growth suppressing effect may occur. [14, 19-22] It seems that especially in puberty the growth suppressing effect of HC may outweigh the negative effect of increased androgen levels.

In childhood however, the A levels and the variability in these levels showed strong negative associations with FH. In our initial analyses, FH was also negatively associated with the salivary 17OHP, but these effects disappeared when glucocorticoid doses were taken into account. Therefore, in childhood the most important risk factor for reduced final height was elevated A levels, in contrast to puberty where the amount of glucocorticoids was more influential.

Based on these results, we suggest that treatment goals should be more specified according to age to find the optimal balance in growth and maturation.

During childhood, the main focus should be on adequately suppressing A levels without completely suppressing 17OHP, thereby preventing an advancement of bone maturation. During puberty, the main aim should be to avoid higher doses of glucocorticoids even when the A levels are not completely suppressed.

Previously, it has been argued that glucocorticoid doses need to be increased during puberty in CAH patients because of the alterations in glucocorticoid metabolism. [4, 23] Our results do not support this hypothesis. In pubertal patients, salivary A levels remained mostly within the age appropriate reference ranges without routinely increasing the HC dose. Moreover, our results suggest that the currently advised doses of glucocorticoids may already have negative effects on final height. Therefore, we suggest being cautious with increasing the dose of glucocorticoid medication in puberty, since the detrimental effects may outweigh the benefits.

In this study, we did not describe growth patterns in infants with CAH. We previously showed that growth velocity and bone maturation were not increased in the first year of life in untreated CAH patients, implying that the growth in the first year is less sensitive to androgens. [24] However, the negative effects of supraphysiological doses of HC may already occur at this age, since it seems likely that these effects are most pronounced when growth velocity is highest. [25, 26] This would lead to a cautious attitude towards glucocorticoid dosing in this age group as well.

Besides the above mentioned strengths of this study, there are some limitations. The study was retrospective in nature and the number of patients was relatively small. Also, the number of available measurements per patients differed considerably. However, by performing mixed model analyses and weighing the linear regression analyses for the square root of the number of measurements, we were able to account for these differences and make full use of all longitudinal data.

In conclusion, the glucocorticoid treatment in pediatric CAH patients should be tailored to the specific phase of growth and development. Salivary steroid measurements in combination with regular measurements of height and BA should be used as tools to optimize treatment. In childhood, the main focus should be on preventing exposure to increased androgens to prevent bone age advancement. In puberty, the attention should be shifted to preventing overtreatment with glucocorticoids.

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Supplemental table

Supplemental table 1 Mutation analysis of the patients included in this study.

Patients per subtype Salt wasting	Mutation allele 1	Mutation group	Mutation allele 2	Mutation group
1	Deletion	0	p.Arg356Trp	0
2	Deletion	0	p.Arg356Trp	0
3	Deletion	0	Δ8bp	0
4	Deletion	0	Δ8bp	0
5	Deletion	0	p.Gln318X	0
6	p.Gln318X	0	p.Val281Leu, p.Gln318X, p.Arg356Trp	0
7	p.Gln318X	0	p.Val281Leu, p.Gln318X, p.Arg356Trp	0
8	Deletion	0	Intron splice	A
9	Deletion	0	p.Arg356Trp	0
10	Deletion	0	Δ8bp	0
11	Deletion	0	p.Arg356Trp	0
12	Deletion	0	E6 cluster	0
13	E6 cluster	0	p.Val281Leu, p.Gln318X, p.Arg356Trp	0
14	Intron splice	0	p.Arg356Trp	0
15	p.Arg356Trp	0	p.Ile172Asn, p.Gln318X, p.Val281Leu, p.Leu307PhefsX6	0
16	p.Arg356Trp	0	p.Arg356Trp	0
17	p.Arg356Trp	0	E6 cluster	0
18	Deletion	0	p.Arg483Pro	A
19	Δ8bp	0	E6 cluster	0
20	Deletion	0	Deletion	0
21	Deletion	0	Deletion	0
22	p.Arg356Trp	0	p.Arg356Trp	0
23[a]	Deletion	0	p.Ile172Asn	B
24	Deletion	0	Intron splice	A
25	Marker analysis prenatally		Marker analysis prenatally	

Supplemental table 1 Continued.

Patients per subtype Simple virilizing	Mutation allele 1	Mutation group	Mutation allele 2	Mutation group
26	p.Ile172Asn	B	p.Ile172Asn	B
27	p.Ile172Asn	B	p.Gln318X	0
28	p.Ile172Asn	B	p.Val237Glu, p.Met239Lys	0
29	Deletion	0	p.Ile172Asn	B
30	p.Ile172Asn	B	Intron splice	A
31	p.Ile172Asn	B	p.Ile172Asn	B
32	p.Ile172Asn	B	p.Ile172Asn	B
33	Deletion	0	p.Ile172Asn	B
34	p.Ile172Asn	B	Δ8bp	0
35	Deletion	0	p.Ile172Asn	B
36	Deletion	0	p.Ile172Asn	B
37[a]	Deletion	0	Deletion	0
38	Deletion	0	p.Ile172Asn	B
39[a]	p.Arg483Pro	A	Intron splice	A

E6 cluster refers to the p.Ile236Asn, p.Val237Glu, and p.Met239Leu mutation cluster at exon 6. Intron splice refers to the c.293–13A/C>G mutation. Δ8bp refers to the p.Gly110Val*fs*X21 mutation.

[a] In three patients, genotype and phenotype were not consistent. These patients were classified according to their phenotype.

Part II

Glucocorticoid and mineralocorticoid effects of adrenal steroid hormone precursors

Chapter 5

A delayed diagnosis of salt wasting congenital adrenal hyperplasia

Pijnenburg-Kleizen KJ, Noordam C, Otten BJ, Claahsen-van der Grinten HL.
Clin Endocrinol. 2016; 85(3): 497-9

In patients with salt wasting congenital adrenal hyperplasia (SW-CAH) due to 21-hydroxylase deficiency synthesis of cortisol and aldosterone is severely impaired. Untreated patients have a high morbidity and mortality due to salt wasting crises neonatally. The lack of cortisol may lead to life-threatening Addisonian crises later in life. We describe a SW-CAH case where the diagnosis and treatment were delayed for several years.

The patient was born before the introduction of neonatal screening for CAH in the Netherlands. He was admitted to the children's department of our hospital at the age of two weeks because of poor feeding, irritability and weight loss. Biochemical analysis showed hyperkalemia (7.5 mmol/l; normal values 3.5-4.7 mmol/l), hyponatremia (132 mmol/l; normal values 135-145 mmol/l) and inadequately high urinary sodium excretion (46 mmol/l; normal values <20 mmol/l). There was no hypoglycemia. Initial treatment consisted of salbutamol, sodium polystyrene sulfonate and intravenous sodium chloride and glucose. Urine analysis showed no signs of a urinary tract infection. Abdominal ultrasound and a voiding cystourethrogram confirmed the presence of posterior urethral valves. The electrolyte abnormalities were explained by secondary pseudohypoaldosteronism (PHA) due to obstructive uropathy. Additional laboratory studies showed an ACTH of 36.0 pmol/l (normal values <11 pmol/l), plasma renin activity of 85 nmol/l/24 h (normal values 1.1 – 20.0 nmol/l/24 h), 17-hydroxyprogesterone (17OHP) of 520.5 nmol/l (normal values 0.2-7.4 nmol/l) and androstenedione of 14.0 nmol/l (normal values 0.21-2.79 nmol/l). The increased concentrations of ACTH, 17OHP and androstenedione were (inappropriately) attributed to adrenal stimulation due to physical stress.

The urethral valves were surgically resected. No glucocorticoids were prescribed during surgery. Afterwards he was discharged home with sodium chloride suppletion orally 30 mmol/day.

During follow-up the sodium chloride dosage was repeatedly increased because of low serum sodium values. At the age of 16 months the patient was readmitted because of an acute otitis media. Serum sodium was 126 mmol/l and serum potassium was 5.9 mmol/l. No measurements of blood glucose or acid/base balance were performed. The hyponatremia was attributed to a decreased intake of sodium and he received extra sodium chloride orally.

At the age of two years he still required sodium chloride supplementation to prevent hyponatremia. Additional laboratory studies were performed: ACTH 13 pmol/l; aldosterone 1.10 nmol/l (normal values 0.1 – 1.7 nmol/l); plasma renin activity 20.20 nmol/l/24 h (normal values 1.0 – 6.4 nmol/l/24 h); cortisol 0.09 umol/l (normal values 0.19-0.55 umol/l); 17OHP 309.80 nmol/l (normal values 0.2-7.4 nmol/l); androstenedione 4.7 nmol/l (normal values 0.03-1.05 nmol/l). He was diagnosed with SW-CAH. The diagnosis was confirmed by mutaton analysis

(homozygous R356W mutation in the *CYP21A2* gene). There were no clinical signs of androgen excess such as an increased growth velocity. He started treatment with hydrocortisone and fludrocortisone. The salt supplementation could be stopped .

It is well known that the clinical presentation of SW-CAH and PHA in the neonatal period overlaps. [1] In both conditions patients develop hyponatremia, hyperkalemia, metabolic acidosis and dehydration due to renal salt wasting. However, the underlying mechanisms of renal salt loss differ. PHA is caused by aldosterone resistance, secondary to urinary tract pathology or due to mutations in the mineralocorticoid receptor or the epithelial sodium channel. [1] In SW-CAH the hyponatremia is caused by a deficiency of aldosterone due to a lack of 21-hydroxylase, an enzyme involved in the synthesis of aldosterone and cortisol. [2] The hyponatremia in our patient was attributed to aldosterone resistance due to his obstructive uropathy, although in hindsight the levels of 17OHP and androstenedione were strongly suggestive of SW-CAH. The normal aldosterone level found in our patient seems to contradict this, however this is most likely the result of cross-reactivity with other, elevated steroids in the laboratory measurement. The patient showed persistent hyponatremia but no clinical signs of cortisol deficiency even during episodes of surgery and acute illness despite a severe *CYP21A2* mutation resulting in no residual enzymatic activity. [3]

We hypothesize that this lack of signs of glucocorticoid deficiency may be partially explained by the presence of elevated concentrations of adrenal steroid hormone precursors that are characteristic in CAH patients . It has previously been shown *in vitro* that 17OHP and progesterone have agonistic effects on the human glucocorticoid receptor and therefore might have glucocorticoid activity. [4] We have recently shown that increased levels of 21-deoxycortisol (21DF) can also transactivate the human glucocorticoid receptor *in vitro* at concentrations of only approximately six times the concentrations of cortisol, which is lower than the concentrations needed for 17OHP and progesterone. [5] Thus 21DF might have glucocorticoid properties closer to the potency of cortisol. 21DF is elevated in 21-hydroxylase deficient CAH patients since it is formed from 17OHP by 11-hydroxylase exclusively in the adrenal cortex in these patients. In contrast to other forms of primary adrenal insufficiency, in CAH patients elevated steroid precursors may partially compensate cortisol deficiency.

Our hypothesis is based on *in vitro* data of which the clinical relevance has not yet been confirmed. There are alternative possible explanations for the lack of signs of cortisol deficiency in some CAH patients. For example, in neonates with CAH a

shift of the balance of cortisol – cortisone interconversion towards cortisol has been demonstrated. [6] This could result in the presence of more active cortisol, if the enzyme deficiency is not complete and there is residual cortisol production. Further studies are necessary to evaluate the role of adrenal steroid precursors in more detail and to determine the clinical relevance.

In conclusion, we present a patient suffering from posterior urethral valves and SW-CAH with a salt wasting crisis but without evident clinical signs of cortisol deficiency. The diagnosis SW-CAH was delayed for two years due to an initial diagnosis of secondary PHA. We hypothesize that increased concentrations of adrenal steroid hormone precursors such as 21DF and 17OHP with agonistic effects on the human glucocorticoid receptor may partially compensate for cortisol deficiency.

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Chapter 6

Adrenal steroid metabolites accumulating in congenital adrenal hyperplasia lead to transactivation of the glucocorticoid receptor

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Abstract

Background Congenital adrenal hyperplasia (CAH) patients are clinically often less severely affected by cortisol deficiency than anticipated from their enzymatic defect. We hypothesize that adrenal steroid hormone precursors that accumulate in untreated or poorly controlled CAH have glucocorticoid activity and partially compensate for cortisol deficiency. We studied the *in vitro* effects of 17-hydroxyprogesterone (17OHP), progesterone (P), 21-deoxycortisol (21DF) and androstenedione (A) on the human glucocorticoid receptor (hGR).

Methods Competitive binding assays were performed in HeLa cells. Nuclear translocation of the hGR was studied by transfection of COS-7 cells with a GFP-tagged hGR and fluorescence microscopy. Transactivation assays were performed in COS-7 cells and in HEK 293 cells after co-transfection with hGR and luciferase reporter vectors using a dual luciferase assay.

Results 17OHP, P and 21DF are able to bind to the hGR with binding affinities of 24 – 43% compared to cortisol. A has a low binding affinity. Incubation with 21DF led to complete nuclear translocation of the hGR, whereas treatment with 17OHP or P resulted in partial nuclear translocation. 21DF transactivated the hGR with an EC50 approximately 6-fold the EC50 of cortisol. 17OHP and P transactivated the hGR with EC50s of more than 100 times the EC50 of cortisol. No hGR transactivation was detected after incubation with A.

Conclusions 21DF, 17OHP and P are able to bind, translocate and transactivate the hGR *in vitro* and thus may have glucocorticoid activity. Mainly 21DF might have a clinically relevant agonistic effect on the hGR and could potentially partially compensate the cortisol deficiency in CAH patients.

Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder caused by a deficiency of one of the enzymes involved in adrenal steroid synthesis. 95% of cases are caused by 21-hydroxylase deficiency. This results in impaired synthesis of cortisol and in 75% of cases also in clinically relevant aldosterone deficiency. [1] Due to the reduced negative feedback to the hypothalamus and pituitary gland, cortisol deficiency leads to increased pituitary secretion of ACTH. Consequently, the steroid hormone precursors prior to the enzymatic block accumulate and are shunted into the androgen-synthesis pathway. The presence of increased concentrations of adrenal steroid hormone precursors is a hallmark feature of patients with CAH and these precursors can be used as diagnostic markers.[2] CAH caused by 21-hydroxylase deficiency represents a spectrum of disease depending on the severity of the enzymatic defect. The most severe, classic CAH is subdivided in a salt wasting form (SW-CAH) and a simple virilizing form (SV-CAH) without aldosterone deficiency. Nonclassic CAH (NC-CAH) is less severe with generally only mild symptoms of hyperandrogenism. [1] The treatment of classic CAH consists of lifelong replacement of glucocorticoids and if necessary also of mineralocorticoids. The goal of treatment is to prevent adrenal and salt wasting crises and to suppress abnormal secretion of adrenal androgens by suppression of the pituitary gland. Treatment of NC-CAH is only indicated when there are severe symptoms of androgen excess and/or glucocorticoid deficiency. [2]

CAH patients are clinically often less severely affected by cortisol deficiency than anticipated from their enzymatic defect. For example, SW-CAH patients have a severe cortisol and aldosterone deficiency due to a residual enzymatic activity of <1%. [1] They develop salt-wasting crises neonatally but only a minority presents with hypoglycemia or conjugated jaundice, a classic symptom of infantile glucocorticoid deficiency. [3] SV-CAH patients have a residual enzymatic activity of 1-2% and a suboptimal increase of cortisol after stimulation with ACTH. In areas where neonatal screening is not implemented, male SV-CAH patients often present with signs of androgen excess in (early) childhood, without a history of Addisonian crises during illness or surgery prior to their diagnosis. [1] Furthermore, many adult patients with classic CAH who are lost to endocrine follow-up and are not adherent to treatment with glucocorticoids do not develop adrenal crises for long periods of time. [4] Finally, between 30 and 40% of patients with NC-CAH diagnosed in adolescence or adulthood show a suboptimal cortisol response to synacthen stimulation suggesting a potential risk of adrenal insufficiency during illness or surgery. [5-7] However, these patients usually do not report a history of signs or symptoms consistent with adrenal insufficiency during surgery or illness. [5,7]

These observations could hypothetically be explained by the presence of adrenal steroid hormone precursors that accumulate in untreated and poorly controlled CAH patients. These precursors, such as 17-hydroxyprogesterone (17OHP), progesterone (P), 21-deoxycortisol (21DF) and androstenedione (A), [2] have structural similarities to cortisol. [8] We hypothesizes that they may have clinically significant glucocorticoid activity and partially substitute for cortisol. To analyze their glucocorticoid properties, we studied their effects on binding, nuclear translocation and transactivation of the human glucocorticoid receptor (hGR).

Materials and methods

In vitro receptor binding assays

Competitive binding assays for the hGR were performed as described previously. [9] HeLa cells were cultured in Dulbecco's minimal essential medium (DMEM) High Glucose (4.5 g/l) with L-Glutamine supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin (all PAA Laboratories GmbH, Pasching, Austria). Whole cells (0.2 – 1.0 x 10^6) were incubated in serum and phenol red free RPMI medium (final volume 150 µl) for 1.5 – 2 hours at 37°C in a series of 0.5 ml microcentrifuge tubes containing 30 nM ^{3}H-cortisol (PerkinElmer Inc, Waltham, MA, USA) and an increasing amount of unlabeled competitor: cortisol, 17OHP, 21DF, A (all Steraloids, Newport, RI, USA) or P (Sigma Aldrich, Gillingham, United Kingdom). Nonspecific binding was assessed by means of 500 fold excess of dexamethasone (Steraloids). Radioactivity was counted in a β counter. Specific binding was expressed as the percentage of specific binding (Bs) over binding of radioligand only (B0), corrected for nonspecific binding.

In vitro nuclear translocation assays

For the intracellular localization assays, hGR DNA (pRShGRα) was PCR amplified using primers with BamHI and EcoRV restriction sites (Sigma Aldrich). It was cloned into a pcDNA6-V5/HisB-EGFP vector (Invitrogen Corp., Carlsbad, CA, USA). The correct insertion of the hGR construct as well as the integrity of the cDNA was checked by direct DNA sequencing.

The assays were carried out in COS-7 cells, which is a cell-line that does not contain endogenous glucocorticoid receptor. The COS-7 cells were cultured in DMEM High Glucose (4.5 g/l) with L-Glutamine supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin. 1.5 x 10^5 COS-7 cells were grown in 6-well plates on glass coverslips. After 24 hours the cells were transiently transfected with 2 µg of GFP-hGR using the TransIT-LT 1 DNA transfection reagent (Mirus Bio, Madison, WI,

USA). 48 hours after transfection the cells were treated for 60 minutes with one of the steroids (cortisol, 17OHP, P, 21DF, A) in a concentration of 10^{-6} M. Afterwards the cells were fixated on the coverslips in 100% methanol at -20°C for 15 minutes and mounted with Vectashield with DAPI (Vector Laboratories, Peterborough, UK) on microscope slides. The cells were studied under the Zeiss Apotome Fluorescence microscope with Zeiss Axiovision imaging software (4.7.2) at a magnification of 200x for the localization of the hGR. Representative images were taken. The experiment was performed in duplicate.

In vitro transactivation assays

For the transactivation assays, the hGR DNA was cloned into a pcDNA6-V5/HisB vector (Invitrogen Corp.) using the same restriction enzymes as described above. The luciferase reporter vectors used for the transactivation assays were MMTV-luc and pRL-TK (Promega, Madison, WI, USA). MMTV-luc is a firefly luciferase reporter construct. Transcription is controlled by glucocorticoid response elements immediately upstream from the luciferase sequence. pRL-TK contains a renilla luciferase. It serves as an internal standard to normalize firefly luciferase light emission measurements with regard to transfection efficiency and the number of cells in each well.

The COS-7 cells were cultured in DMEM High Glucose (4.5 g/l) with L-Glutamine supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin and seeded in 24-well plates in a density of 2 x 10^4 cells per well. Twenty-four hours after seeding, the COS-7 cells were transiently co-transfected using the TransIT-LT 1 DNA transfection reagent with 0.2 µg pcDNA6-V5/HisB-hGR, 0.3 µg MMTV-luc and 0.01 µg pRL-TK per well. Two days after transfection the cells were treated with one of the steroids (cortisol, 17OHP, P, 21DF, A). Steroid solutions in ethanol were made in concentrations of 200x the final desired concentration (10^{-9} – 10^{-4} M) and diluted 1:200 with DMEM prior to adding them to the transfected cells. Twenty-four hours after adding the steroid, firefly and renilla luciferase activity were measured using the Dual-Luciferase Reporter Assay System (Promega) on a Fluoroskan FL luminometer (Thermo Scientific, Franklin, MA, USA). Firefly luciferase/renilla luciferase ratios were calculated to normalize for transfection efficiency. Each experiment was performed in triplicate.

To ensure that the COS-7 cell line did not contain relevant amounts of endogenous steroid receptors, the transactivation activities of the different steroids were also measured in COS-7 cells that had not been transfected with steroid receptor expression vectors. Likewise, the system was tested for the presence of endogenous steroids by measuring the transactivation activity after transfection with the hGR

vectors but without addition of steroids. In neither approach relevant transactivation was measured.

The transactivation assay was repeated in HEK293 cells using the methods described above. These experiments were performed in duplicate.

Statistical analysis

Analyses were performed using GraphPad Prism software version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Steroid concentrations were expressed on a log scale and dose-response curves were calculated using non-linear regression. For the receptor binding assays, the concentration of unlabeled steroid that reduces binding of the radioligand by half (IC50) was determined. The relative binding affinity of the study steroids compared to cortisol was calculated by $IC50_{cortisol}$ / $IC50_{test\ steroid}$ x 100%. For the transactivation assays, the estimated concentration for 50% transactivation (EC50) was determined. For calculation of the relative functional sensitivity of the hGR to the different steroids, the transactivation potential of cortisol was set at 100%. The sensitivity of the hGR to the other test compounds was calculated as $EC50_{cortisol}$ / $EC50_{test\ steroid}$ x 100%.

Results

Receptor binding assay

The receptor binding curves for the studied steroids are shown in Figure 1. The IC50 and the relative binding affinity of cortisol, 17OHP, P, 21DF and A were calculated (Table 1). The results demonstrate that 17OHP, P and 21DF bind to the hGR with relative binding affinities of 27%, 43% and 24%, respectively. The binding affinity for A is dramatically less than that of cortisol, 0.4%.

Nuclear translocation assay

Incubation with cortisol in a concentration of 10^{-6} M resulted in complete nuclear translocation of the hGR within 60 minutes (Figure 2b). Adding 17OHP, P and 21DF to the transfected cells in a concentration of 10^{-6} M resulted in respectively almost complete, partial and complete transport to the nucleus (Figure 2c-e). After treatment of the cells with A, the location of the hGR was still predominantly cytoplasmic (Figure 2f).

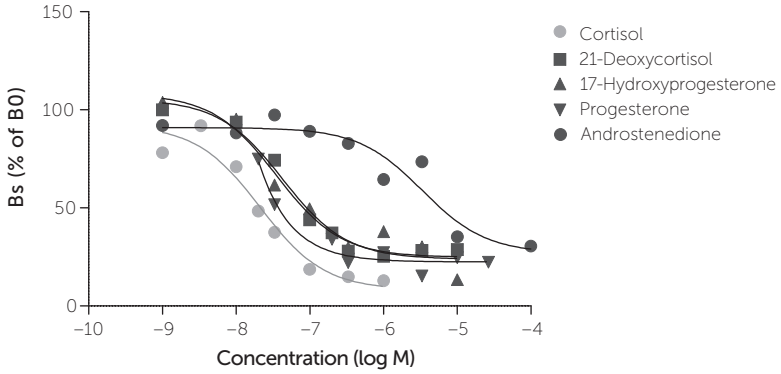

Figure 1 Binding assays. Competition of various steroids for binding of 3H cortisol to the hGR in HeLa cells.

Binding data are expressed as the percentage of specific binding (Bs) remaining after adding increasing amounts of competitor.

hGR transactivation assay in COS-7 cells

Cortisol activated the hGR with an EC50 of 1.7 x 10^{-8} M (Figure 3a-d, Table 1). Exposure of the hGR to increasing concentrations of 17OHP, P and 21DF resulted in increasing hGR transactivation, up to a maximum. A dose-response curve was fitted (Figure 3a-c) and the EC50 values for these steroids were calculated. The EC50 value was 2.2 x 10^{-6} M for 17OHP, 3.0 x 10^{-6} M for P and 1.0 x 10^{-7} M for 21DF (Table 1). A did not transactivate the hGR at the concentrations tested (Figure 3d).

hGR transactivation assay in HEK293 cells

The results for the transactivation assay in HEK293 cells are shown in Supplementary Figure 1 and Table 1. In these experiments, the EC50 for cortisol was 3,5 x 10^{-9} M. 17OHP, P and 21DF activated the hGR with EC50 values of 1.2 x 10^{-6} M, 2.3 x 10^{-6} M and 4.1 x 10^{-8} M respectively.

Table 1 Binding and transactivation capacity of cortisol, 17-hydroxyprogesterone (17OHP), progesterone, 21-deoxycortisol (21DF) and androstenedione to the hGR.

	Binding assay		Transactivation assay in COS-7 cells		Transactivation assay in HEK293 cells	
	IC50 (95% confidence interval)	Relative binding affinity	EC50 (95% confidence interval)	Relative functional sensitivity	EC50 (95% confidence interval)	Relative functional sensitivity
Cortisol	2.2×10^{-8} ($1.3 \times 10^{-8} – 3.8 \times 10^{-8}$)	100 %	1.7×10^{-8} ($1.0 \times 10^{-8} – 2.8 \times 10^{-8}$)	100 %	3.5×10^{-9} ($1.8 \times 10^{-9} – 6.9 \times 10^{-9}$)	100 %
17OHP	8.2×10^{-8} ($3.8 \times 10^{-8} – 1.6 \times 10^{-7}$)	27 %	2.2×10^{-6} ($1.5 \times 10^{-6} – 3.2 \times 10^{-6}$)	0.8 %	1.2×10^{-6} ($7.6 \times 10^{-7} – 1.9 \times 10^{-6}$)	0.3 %
Progesterone	5.1×10^{-8} ($3.1 \times 10^{-8} – 7.8 \times 10^{-8}$)	43 %	3.0×10^{-6} ($1.5 \times 10^{-6} – 5.9 \times 10^{-6}$)	0.6 %	2.3×10^{-6} ($1.2 \times 10^{-6} – 4.8 \times 10^{-6}$)	0.2 %
21DF	9.1×10^{-8} ($6.2 \times 10^{-8} – 1.3 \times 10^{-7}$)	24 %	1.0×10^{-7} ($5.2 \times 10^{-8} – 2.0 \times 10^{-7}$)	17 %	4.1×10^{-8} ($2.1 \times 10^{-8} – 8.2 \times 10^{-8}$)	8.5 %
Androstenedione	5.9×10^{-6} ($2.0 \times 10^{-6} – 2.2 \times 10^{-5}$)	0.4 %	-	-	-	-

IC50: estimated concentration that reduces binding of the radioligand by 50%, in mol/liter.
Relative binding affinity: the binding affinity of cortisol is set at 100%. The binding of the other steroids to the hGR is calculated as $IC50_{cortisol}$ / $IC50_{test\ steroid}$ x 100%.
EC50: estimated concentration for 50% transactivation, in mol/liter.
Relative functional sensitivity: the transactivation potential of cortisol is set at 100%. The sensitivity of the hGR to the other test compounds is calculated as $EC50_{cortisol}$ / $EC50_{test\ steroid}$ x 100%.

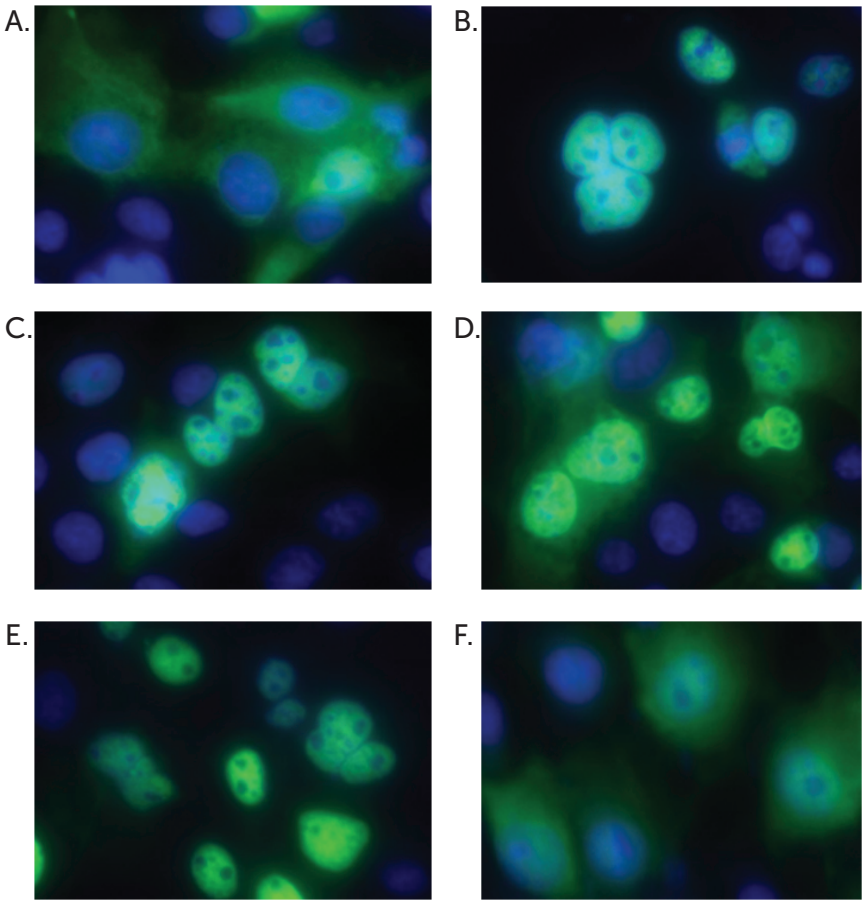

Figure 2 Localization of the human glucocorticoïd receptor (hGR) in COS-7 cells transfected with the hGR, without steroids (A) and after incubation with various steroids in a concentration of 10^{-6} M (B-F).

The nucleus is stained blue, the hGR is tagged with a Green Fluorescent Protein. B cortisol. C 17-hydroxy-progesterone. D progesterone. E 21-deoxycortisol. F androstenedione.

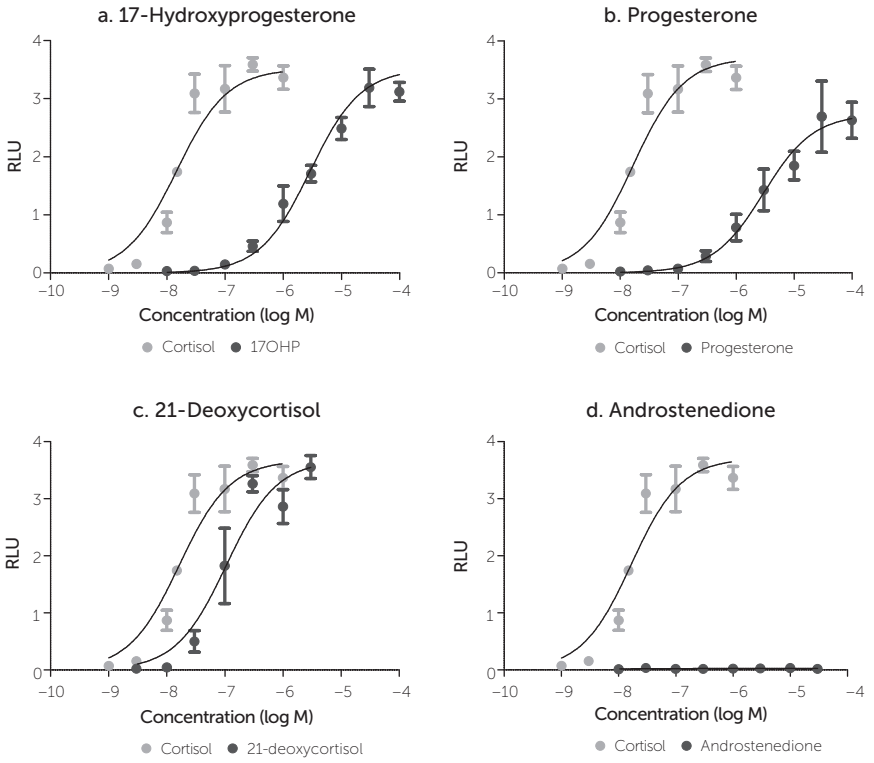

Figure 3 Transactivation of the human glucocorticoid receptor (hGR) by 17-hydroxyprogesterone, progesterone, 21-deoxycortisol and androstenedione, in comparison with the transactivation of the hGR by cortisol.

Discussion

We here describe the binding of 17OHP, P, 21DF and A to the hGR and their effects on the nuclear translocation and transactivation of the hGR, in comparison to the effects of cortisol. We found that 21DF and to a lesser extent 17OHP and P can transactivate the hGR. This is consistent with the receptor binding and nuclear translocation of the hGR we found after incubation with these steroids. It has previously been described that 17OHP and P bind to the hGR [10-12], but to the best of our knowledge this has not been shown for 21DF previously. These agonistic properties on the hGR might explain the clinical observation that CAH patients are often less severely affected by cortisol deficiency than anticipated from their enzymatic defect.

Steroid hormone cross-reactivity to receptors is a well-known phenomenon. For example, cortisol is known to have an agonistic effect on the mineralocorticoid receptor. [13] Additionally, in a previous study of our group we have demonstrated that 17OHP and P can influence the transactivation of the human mineralocorticoid receptor (hMR): both have anti-mineralocorticoid effects *in vitro*. [14] The occurrence of cross-reactivity of these steroids at both the hGR and the hMR can be explained by the high degree of homology at the DNA-binding domains of these receptors. [15]

The EC50 of cortisol in our model is comparable to the EC50 previously described [16], demonstrating the reliability of our *in vitro* model. Under the experimental conditions 21DF, 17OHP and P have the capacity to transactivate the hGR. With the concentrations used in our experiments we were able to reconstruct complete dose response curves, up to the point where maximum transactivation was reached. In the two cell lines studied, a comparable profile was found: 21DF has the greatest transactivational capacity, 17OHP and progesterone are also able to transactivate the hGR but only at much higher concentrations. A does not transactivate the hGR at the concentrations tested. Since there was no transactivation detectable for A even in concentrations at least 100 times higher than the concentrations found in untreated CAH, we do not expect relevant transactivation will occur with higher concentrations. [17] The fact that A has no agonistic effect on the hGR might be explained by the greater structural dissimilarity between A and cortisol compared to that of the other test compounds and cortisol. [8]

As illustrated by the EC50 values more than a hundred times higher than the EC50 of cortisol, 17OHP and progesterone are less potent agonists of the hGR than cortisol. In healthy volunteers the 17OHP concentration is substantially lower than that of cortisol: reference values for serum 17OHP are 2.0-10.8 nmol/L for males and 0.45 – 12.7 nmol/L for females, 9 AM reference values for serum cortisol are 190 – 550 nmol/L. In this situation cross-reactivity on the hGR will be negligible. However, in classic CAH patients 17OHP concentrations can increase to very high concentrations of more than 1500 nmol/l. [18,19] Based on the dose-response curve we constructed, these concentrations might be high enough to result in relevant hGR transactivation.

Interestingly, the EC50 of 21DF is much closer to the EC50 of cortisol (approximately six-fold in the transactivation assays in COS7 cells, approximately twelve-fold in the HEK293 cells). The serum concentrations of 21DF in untreated or poorly controlled patients with classic CAH can exceed 400 nmol/l. [18,19] Based on our results we hypothesize that these concentrations may lead to a clinically relevant transactivation

of the hGR. Less transactivation can be expected in NC-CAH patients, since in these patients the 21DF concentrations are lower and reach up to approximately 40 nmol/l [6]. We suggest that high serum 21DF concentrations in untreated or poorly controlled CAH patients may partially compensate for their cortisol deficiency. In contrast, overtreatment with complete suppression of adrenal precursors including 17OHP and 21DF might lead to an increased risk of adrenal crises and hypoglycemia. Therefore, adequate stress dosing is crucial once glucocorticoid treatment is initiated in CAH patients.

Considering that we have studied several elements of the hGR transactivation cascade with consistent results, we are confident that our results represent actual glucocorticoid properties of 21DF, 17OHP and P. However, since this is an *in vitro* model, our findings need to be confirmed in further studies. We have studied the transactivating properties of several steroids, and not the other mechanisms by which the glucocorticoid receptor exerts its actions: transrepression and nongenomic effects. [13,20,21] It might also be relevant to study other steroid precursors. In spite of these limitations, we consider our results promising and potentially relevant for a significant group of CAH patients.

In conclusion, 21DF and to a lesser extent 17OHP and P are able to transactivate the hGR in vitro and thus may have glucocorticoid activity. 21DF, which can be strongly elevated in untreated or poorly controlled CAH patients, has the strongest agonistic effect on the hGR and may partially compensate for the cortisol deficiency in CAH patients.

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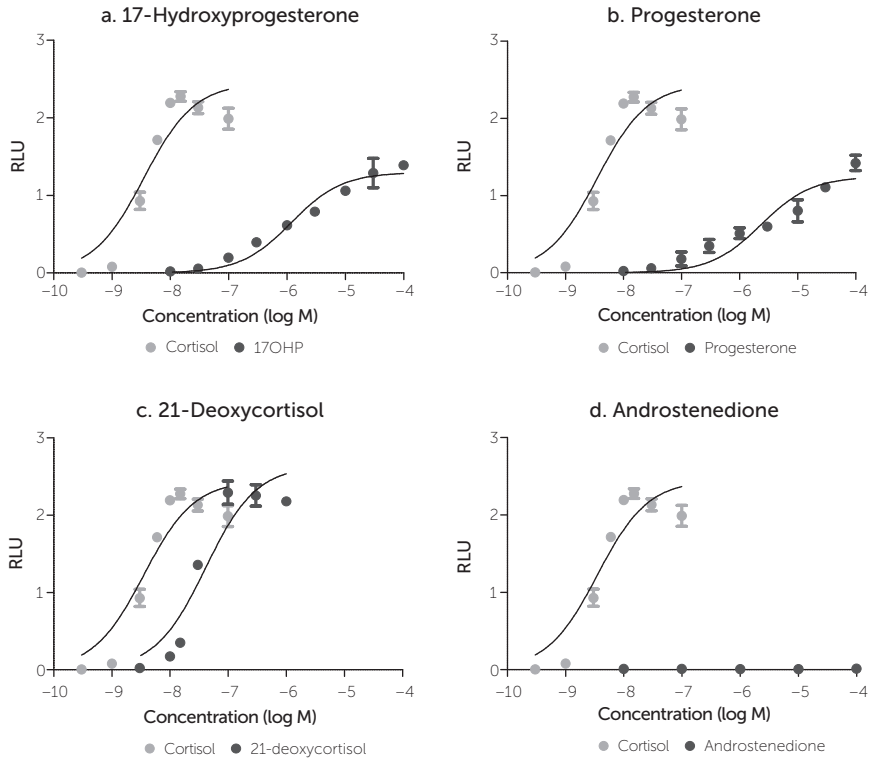
Supplementary Figure



Supplementary figure 1 Transactivation of the human glucocorticoid receptor (hGR) in HEK293 cells by 17-hydroxyprogesterone, progesterone, 21-deoxycortisol and androstenedione, in comparison with the transactivation of the hGR by cortisol.

Chapter 7

Glucocorticoid activity of adrenal steroid precursors may prevent adrenal crises in untreated congenital adrenal hyperplasia patients

Engels M, Pijnenburg-Kleizen KJ, Utari A, Faradz SMH, Oude-Alink S, van Herwaarden AE, Span PN, Sweep CGJ, Claahsen - van der Grinten HL. Submitted.

Abstract

Background Cortisol deficiency can cause adrenal crises, which can be life-threatening. Cortisol production is impaired in patients with congenital adrenal hyperplasia (CAH). Here, we describe a unique population of severely affected untreated CAH patients, with proven cortisol deficiency but without clinical signs of cortisol deficiency even in severe stress-situations. The lack of clinical signs of cortisol deficiency in untreated CAH patients might be explained by the glucocorticoid activity of increased concentrations of adrenal steroid precursors.

Methods Adrenal steroid precursor concentrations before and 60 minutes after ACTH administration of 22 untreated Indonesian CAH patients (3-46 years) with proven cortisol deficiency (< 500nmol/l post-ACTH) measured by liquid chromatography tandem-mass spectrometry (LC-MS/MS) were compared to 6 control patients (Mann-Whitney U test). Glucocorticoid activity was determined by dual-luciferase assays in human embryonic kidney cells transfected with the glucocorticoid receptor and exposed to increasing amounts of adrenal steroid precursors for 24 hours.

Results Blood concentrations of the steroid precursors 11-deoxycortisol (457nmol/l, $p=0.003$), 11-deoxycorticosterone (55nmol/l, $p=0.003$), 17-hydroxyprogesterone (610nmol/l , $p<0.001$), progesterone (29nmol/l, $p<0.001$), and 21-deoxycortisol (73 nmol/l) were strongly elevated compared to controls. The hGR was activated with comparable potency to cortisol by corticosterone and 21-deoxycortisol, and with 4-100x lower potency by 11-hydroxyprogesterone, 11-deoxycortisol, aldosterone, 11-deoxycorticosterone, progesterone, and 17-hydroxyprogesterone.

Conclusions We identified strongly elevated adrenal steroid precursor concentrations in blood from untreated CAH patients and demonstrated glucocorticoid activity of these adrenal precursors *in vitro* – implicating these precursors might be able to compensate for cortisol deficiency in untreated CAH patients, thereby potentially protecting against life-threatening adrenal crises.

Introduction

An adrenal crisis due to cortisol deficiency, also known as Addisonian crisis, is a life-threatening condition when not properly treated with glucocorticoids. It is often described by its main symptoms: vomiting, diarrhea, and abdominal pain. Whereas the main cause of an adrenal crisis is cortisol deficiency, additional aldosterone deficiency can lead to the development of a salt wasting crisis, which is treated by supplementation of mineralocorticoids and sodium chloride.

Cortisol deficiency is one of the main findings in patients with congenital adrenal hyperplasia (CAH). CAH is an autosomal recessive disorder with impaired steroidogenesis in the adrenal cortex. Adrenal steroidogenesis involves the synthesis of cortisol, aldosterone, and androgens from the common precursor cholesterol via several enzymatic steps. More than 90% of CAH cases are caused by a mutation in the *CYP21A2* gene resulting in 21-hydroxylase deficiency (21OHD). In 21OHD, conversion of the steroid precursors 17-hydroxyprogesterone (17OHP) and progesterone to 11-deoxycortisol and 11-deoxycorticosterone respectively is impaired (Figure 1). Consequently, cortisol production is inadequate, leading to elevated ACTH concentrations due to a lack of negative feedback to the hypothalamus and pituitary gland. Increased ACTH concentrations will stimulate the adrenal gland, eventually causing hyperplasia. Furthermore, adrenal steroid precursors before the enzymatic defect accumulate, and are shunted into the androgen synthesis pathway. [1] Increased concentrations of adrenal steroid precursors 17OHP and 21-deoxycortisol (21DF) are currently used as diagnostic markers for CAH. [1-3] The severity of the disease depends on the residual enzymatic activity with a strong phenotype-genotype relationship: Mutations that are classified as group null (0) or A are usually associated with <1% residual enzyme activity with additional aldosterone deficiency (salt wasting type), group B with 1-5% residual enzyme activity without aldosterone deficiency (simple virilizing type), and group C with a residual enzymatic activity of 20-50% and a less severe phenotype with usually normal cortisol and aldosterone levels (non classic type). [4]

Approximately 5% of CAH cases are caused by mutations in the *CYP11B1* gene resulting in 11-hydroxylase deficiency (11OHD). 11OHD results in decreased conversion of the precursors 11-deoxycorticosterone and 11-deoxycortisol to corticosterone and cortisol, respectively (Figure 1). As in 21OHD, cortisol production is deficient and adrenal androgen concentrations are increased. However, in contrast to 21OHD, signs of mineralocorticoid excess occur with hypertension and hypokalemia due to the mineralocorticoid potency of 11-deoxycorticosterone. [5]

Patients with cortisol deficiency are generally treated with glucocorticoids to substitute for cortisol deficiency in order to prevent life-threatening adrenal crises and to suppress the elevated androgens. In our clinical experience however, there

are cases of untreated or noncompliant severely affected CAH patients known to have less clinical signs of cortisol deficiency than expected based on their enzyme deficiency, and compared to patients with other forms of adrenal insufficiency. For example, only a minority of neonates with the most severe form of 21OHD present with typical features of cortisol deficiency (hypoglycemia or conjugated jaundice). [6] We have previously described a patient with salt wasting CAH, who was only treated with sodium chloride for the first two years of life, without apparent complications from cortisol deficiency. [7] Moreover, in countries without neonatal screening programs for CAH, CAH patients from group B (simple virilizing) usually only present with signs of androgen excess during childhood, without reporting a history of adrenal crises.

The aim of our study was to determine if this lack of signs of cortisol deficiency might be explained by the glucocorticoid activity of increased concentrations of adrenal steroid precursors that accumulate before the enzymatic defect. Here, we describe the clinical phenotype and the biochemical profile of adrenal steroid precursors in a unique cohort of untreated 21OHD and 11OHD CAH patients, before and after ACTH administration using liquid chromatography tandem-mass spectrometry (LC-MS/MS). The glucocorticoid activity of these precursor steroids was assessed by *in vitro* human glucocorticoid receptor (hGR) transactivation studies.

Patients and methods

Study design

Within this translational study we had the objective to describe the phenotype and steroid profile of a cohort of untreated Indonesian CAH patients. No sample size calculation was performed in this descriptive study. We added 6 control patients, and 2 untreated CAH patients from the Netherlands in comparative biochemical analyses. Our inclusion criteria were untreated patients with a severe type of genetically confirmed CAH in which an ACTH stimulation test was performed. To understand the potential of different steroid hormones, we also performed cell culture experiments. We determined the potency to activate the hGR for each steroid, in which each concentration was measured in triplicate. No rules for stopping data collections were defined. We reported all data and did not find outliers in our data. Main outcomes in our study were defined as the number of stress-full events, biochemical evaluation of the ACTH stimulations tests by LC-MS/MS and activation of hGR.

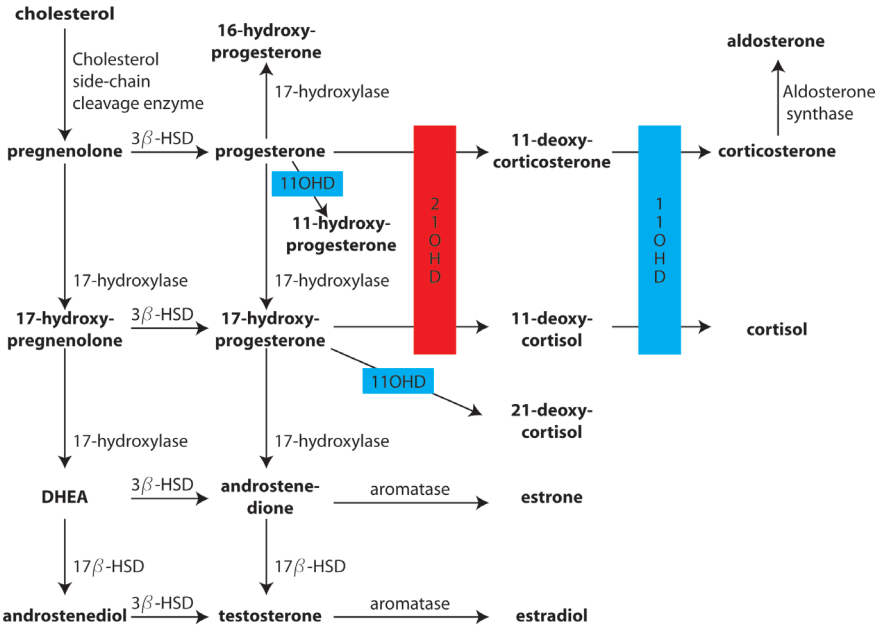


Figure 1 Adrenal steroidogenesis. Cholesterol is converted to aldosterone, cortisol and androgens in the adrenal cortex via several enzymatic steps.

Abbreviations: 11OHD, 11-hydroxylase deficiency; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 21OHD, 21-hydroxylase deficiency; 3β-HSD, 3β-hydroxysteroid dehydrogenase.

Patients

Untreated CAH patients (n=22) were recruited from the local CAH database and included as a part of a cohort study on hormonal analysis and compliance in the Center for Biomedical Research, Faculty of Medicine Diponegoro University, Semarang, Indonesia (in compliance with relevant laws, institutional guidelines and the declaration of Helsinki). The study was approved by the local ethical committee of the Diponegoro University. Oral and written informed consent was obtained after full explanation of the purpose and nature of all procedures. Data were collected on the type of CAH, karyotype, gender, mutation analysis, signs of salt wasting, and episodes of severe stress in their medical history (critical illness, trauma, surgery) by the local pediatric endocrinologist (AU). For all 21OHD patients, the severity of CAH was classified based on their mutation analysis into genotype groups 0, A, B, or C (4).

In addition, the biochemical results of patients in whom an ACTH test was performed at Radboud university medical center in Nijmegen, the Netherlands in 2017 were included (n=8). Oral informed consent was obtained by the treating physician as additional measurements were performed on coded stored blood samples in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.federa.org/codes-conduct). Two patients had a CAH diagnosis, the other 6 were non-CAH patients, falsely suspected of an adrenal disorder and were used as control group. Three of these patients were females with polycystic ovarian syndrome. One male patient presented with an abnormal result in the neonatal screening program for CAH, which turned out to be false positive, and the 2 other patients were 46XY neonates with ambiguous genitalia. In one of them, no final diagnosis could be made. In the other patient, a mutation with unknown pathogenicity was found in the NR5A1 gene. Additionally, 1 healthy adult female underwent an ACTH stimulation test in the Center for Biomedical Research, Faculty of Medicine Diponegoro University, Semarang, Indonesia to serve as a control patient.

Biochemical analysis

The standard ACTH stimulation test was performed in all patients to determine adrenal functioning by measuring steroid concentrations before and after ACTH administration. Synacthen (0.25 mg; Sigma Tau BV) was injected intravenously, with blood draws for steroid analysis before and 60 minutes after the injection. In the Indonesian patients all tests were performed before 9.00 am. Dutch patients had their ACTH stimulation test during their clinical appointment, which mostly took place in the morning. None of the patients received glucocorticoids at the time of biochemical analysis. Serum before and after ACTH administration were analyzed with LC-MS/MS to determine cortisol, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, 17OHP, progesterone, and 21DF concentrations. The LC-MS/MS protocol has been provided in the Supplemental Material.

In vitro transactivation study

Cell culture

Human embryonic kidney cells (HEK293) were grown as a monolayer culture in DMEM with 4.5 g/L glucose with l-glutamine (Lonza; Leusden, the Netherlands) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific; Landsmeer, the Netherlands) and 1% antibiotics (penicillin-streptomycin 10,000 U/mL; Gibco). Cells were cultured at 37 °C in a humidified 95% air / 5% CO2 atmosphere, and passaged when confluent.

In vitro dual luciferase transactivation assays

Human hGR transactivation was measured using dual-luciferase transactivation assays (Promega; Leiden, the Netherlands) in which pcDNA6-V5/Hisb-hGR, MMTV-luc, and pRL-TK vectors were used as described earlier. [8]

HEK293 cells were seeded at 40,000 cells/well in 24-well plates. Transient transfection was performed after 24 hours, using 0.2 µg pcDNA6-V5/Hisb-hGR, 0.3 µg MMTV-luc, and 0.01 µg pRL-TK per well and 1 µL TransIT-LT1 transfection reagent (Mirus; Ochten, the Netherlands) according to manufacturer's protocol. Cells were treated for 24 hours with one of the steroids (progesterone, 17-hydroxypregnenolone, pregnenolone (Sigma-Aldrich, Zwijndrecht, the Netherlands) or cortisol, aldosterone, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, 17OHP, 11β-hydroxyprogesterone, 16-hydroxyprogesterone, 21DF, (Steraloids, Newport, USA)) 2 days after transfection. Steroid solutions were prepared in ethanol (200X concentrated) and diluted 1:200 in culture medium prior to treatment. Thereafter, firefly and renilla luciferase activity was measured on a Fluoroskan FL luminometer (Thermo Scientific) according to manufacturer's protocol (Promega) and firefly/renilla ratios were calculated. Each concentration was measured in triplicate.

As controls, HEK293 cells were transfected with the MMTV-luc and pRL-TK vector, but not the hGR and treated with 10^{-4} M cortisol. Secondly, HEK293 cells were transfected with all the vectors, but not treated with any steroid (medium with 0.5% ethanol). Neither of these approached resulted in transactivation activity.

Statistical analyses

GraphPad Prism software version 5.0 for Windows and SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA) were used to analyze adrenal steroid concentrations. Normality was assessed and median steroid concentration and interquartile range (IQR: Q1-Q3) were calculated. 21OHD and 11OHD patients were compared to the control group and distributions were compared using the Mann-Whitney U (MWU) test. $P<0.05$ was considered significant. GraphPad Prism was also used to calculate dose-response curves using nonlinear regression. The estimated concentration that causes 50% of the maximum transactivation (EC50) and its 95% confidence interval (95%CI) were also determined. Relative functional sensitivity of the hGR to the different steroids was calculated as $EC50_{\text{cortisol}} / EC50_{\text{test steroid}} \times 100\%$ in which cortisol was set as 100%.

Results

Clinical characteristics of untreated CAH patients

Clinical characteristics of the included CAH patients are shown in Table 1. Twenty-two (long-time) untreated CAH patients were identified in the Indonesian CAH database, age 3-46 years, of which 17 had 21OHD and 5 had 11OHD. Most patients (n=15) were never treated with glucocorticoids, and 7 patients were treated in the past, but had been off treatment for at least 2 years at the time of biochemical analysis and were therefore considered as untreated. Additionally, we included the biochemical results of 2 Dutch male CAH neonates, who were identified from the national CAH neonatal screening program, and started glucocorticoid treatment only after the biochemical analysis of the ACTH stimulation test. All patients were diagnosed with a severe type of CAH based on mutation analysis. Episodes of severe stress in their medical history has been reported in 13/22 patients. In none of these episodes patients were treated with stress dosages of glucocorticoids. In the 11OHD group, 1 patient underwent genital surgery. In the 21OHD group, 5 patients underwent genital surgery (genotype group 0 (n=3), A (n=1), B (n=1)) and 2 patients underwent tonsillectomy (genotype group 0 (n=1), unclassifiable (n=1)). There were 5 reports of dengue hemorrhagic fever (genotype group 0 (n=2), unclassifiable (n=1)) or typhoid fever (genotype group 0 (n=1), B (n=1). Also, 5 patients reported hospital admissions because of salt wasting crisis (genotype group A (n=2)) or because of episodes of vomiting and/or seizures (genotype group 0 (n=3)).

Biochemical evaluation of serum adrenal steroid precursors

In control patients, cortisol concentration increased adequately after ACTH administration (>500 nmol/l (9-11)), and all other measured steroids remained low with a 2-5 fold increase. In contrast, in our study population cortisol concentrations remained low after ACTH administration with a median concentration of 73 (IQR 64-129) nmol/l in the 21OHD patients and 180 (IQR 159-214) nmol/l in the 11OHD patients, confirming severe cortisol deficiency in our cohort (Table 1, Figure 2). Adrenal steroid precursor concentrations were also measured in serum from CAH patients before and after ACTH administration (Figure 2). As we did not find differences in steroid concentrations before and after ACTH administration, we further only report on the steroid concentrations in serum after ACTH administration. Corticosterone concentrations were significantly lower in untreated CAH patients (median 21OHD: 7.1 (IQR 5.9-8.2) nmol/l, MWU $p<0.001$; median 11OHD: 4.3 (IQR 3.7-8.4) nmol/l, MWU $p=0.003$) compared to controls (median 72 (IQR 55-78) nmol/l). Blood 11-deoxycortisol and 11-deoxycorticosterone concentrations were significantly elevated in 11OHD patients with respectively a median concentration

of 457 nmol/l (IQR 364-612, MWU p=0.003) and 55 nmol/l (IQR 25-119, MWU p=0.003) compared to control (median 3.2 (IQR 1.9-5.9), and 0.5 (IQR 0.2-0.8) nmol/l, respectively), but not in 21OHD patients (median 1.9 (IQR 1.5-3.8, MWU p=0.083) and 0.4 (IQR 0.3-0.6, MWU p=0.395) nmol/l, respectively). Blood 17OHP and progesterone concentrations were elevated in 21OHD patients (median 610 (IQR 509-762, MWU p<0.001) and 29 (IQR 20-43, MWU p<0.001) nmol/l, respectively) and 11OHD patients (median 20 (IQR 16-21, MWU p=0.003), and 3.6 (IQR 2.6-5.8, MWU p=0.003) nmol/l, respectively) compared to controls (median 4.8 (IQR 2.7-5.6) nmol/l and 1.0 (IQR 0.5-1.1) nmol/l, respectively). 21DF concentrations were only measurable (>1 nmol/l) in blood from untreated 21OHD patients (median 73 (IQR 46-112) nmol/l).

hGR transactivation in human embryonic kidney cells

To determine the potency of steroid precursors for hGR transactivation, we performed dual luciferase assays, which allows for the quantification of hGR-induced gene expression (Table 2, Figure 3). We found an EC50 of 11 nM (95%CI: 5.9-20) for cortisol, which is used as reference steroid. Similar potencies were found for corticosterone (EC50 of 17 nM, 95%CI: 8.9-32) and 21DF (EC50 of 22 nM, 95%CI: 12-42) as confidence intervals overlap.

11-hydroxyprogesterone, 11-deoxycortisol, and aldosterone exhibited somewhat higher EC50 values: 47, 71, and 111 nM, respectively. Exposure to 11-deoxycorticosterone (EC50 of 725 nM), progesterone (EC_{50} of 1,147 nM), and 17OHP (EC50 of 1,668 nM) also resulted in hGR transactivation, although these EC50 values were at least 65 times higher than that of cortisol. hGR transactivation was only observed at a very high concentration (100,000 nM) of 16-hydroxyprogesterone, while no hGR transactivation was found for pregnenolone, and 17-hydroxypregnenolone.

Table 1 Clinical characteristics of untreated CAH patients.

Patient	Age	CAH type	Karyotype	Gender	Mutations in CYP11B1 (#I1-I5) and CYP21A2 (#I6-D2)	Geno-type group ^
I1	28	11OHD	46XX	F	c.799G>A c.799G>A	NA
I2	19	11OHD	46XX	F	c.799G>A c.799G>A	NA
I3	5	11OHD	46XX	M	c.799G>A c.799G>A	NA
I4	13	11OHD	46XX	M	c.799G>A c.799G>A	NA
I5	3	11OHD	46XX	U	c.799G>A c.799G>A	NA
I6	19	21OHD	46XX	F	p.Ile172Asn p.Ile172Asn	B
I7	23	21OHD	46XX	F	p.Ile172Asn p.Ile172Asn	B
I8	21	21OHD	46XX	F	p.Arg356Trp p.Arg356Trp	0
I9	9	21OHD	46XX	F	intron splice p.Arg356Trp	A
I10	4	21OHD	46XX	F	intron splice p.Arg356Trp	A
I11	13	21OHD	46XX	F	p.Arg356Trp p.Arg356Trp	0
I12	46	21OHD	46XX	M	p.Trp20* p.Ile172Asn	B
I13	15	21OHD	46XX	M	intron splice intron splice	A
I14	32	21OHD	46XX	M	intron splice intron splice	A
I15	13	21OHD	46XX	F	p.Arg356Trp p.Arg356Trp	0
I16	15	21OHD	46XX	M	p.Arg356Trp deletion of exon 1-3	0
I17	14	21OHD	46XX	M	p.Ile386del p.Arg356Trp	0
I18	14	21OHD	46XX	F	p.Pro30Leu p.Pro30Leu	B

Treatment with glucocorticoids	History of salt wasting	History of severe stress while untreated	cortisol before ACTH	cortisol after ACTH
never	no	genital surgery	164	163
never	no	no	218	225
never	no	no	150	155
never	no	no	179	180
never	no	no	202	202
treated at age 8-16 yr stopped >3 yrs	no	no	110	125
treated at age 13-20 yr stopped >3 yrs	no	no	233	238
treated at age 10-19 yr stopped >2 yrs	yes	genital surgery	68	66
treated at age 5-7 yr stopped >2 yrs	yes	repeated hospital admissions for salt wasting crises	82	73
treated at age 0-2 yr stopped >2 yrs	yes	repeated hospital admissions for salt wasting crises	35	33
treated since childhood stopped > 4 years	no	genital surgery	115	119
never	no	severe gastritis (2x), typhoid fever	159	159
never	no	no	89	79
never	no	genital surgery	61	65
treated for one year at age 5 yr stopped > 8 yrs	no	genital surgery, tonsillectomy, dengue hemorrhagic fever, typhoid fever	75	68
never	yes	repeated hospital admissions for vomiting and seizures	57	58
never	yes	repeated hospital admissions for seizures	53	50
treated at age 4-10 yr stopped > 4 yrs	no	genital surgery	210	187

Table 1 Continued.

Patient	Age	CAH type	Karyotype	Gender	Mutations in CYP11B1 (#I1-I5) and CYP21A2 (#I6-D2)	Geno-type group ^
I19	10	21OHD	46XX	M	p.Gln196* p.Arg356Trp	0
I20	19	21OHD	46XX	F	p.Ile172Asn deletion of exon 1-6	B
I21	3	21OHD	46XX	F	p.Ile172Asn p.Arg356Trp	B
I22	21	21OHD	46XX	F	deletion of exon 1-6 no second mutation #	NC
D1	0	21OHD	46XY	M	deletion of exon 1-3 p.Ile172Asn	B
D2	0	21OHD	46XY	M	deletion of exon 1-7 p.Pro30Leu	B

^Genotype classification is based on mutation analysis. Mutations in genotype group 0 and A usually have <1% residual enzyme activity, genotype B has 1-5% residual enzyme activity, while genotype C has a residual enzyme activity of 20-50% (4). We classified the p.Pro30Leu mutation in genotype group B. * This patients also had a cluster of mutations in the CYP21A2 promotor region in one allele, possibly resulting in a more severe phenotype. # This patient had very high 17OHP levels (>400 nmol/l), and although no second mutation has been found it is very likely that this patient belongs to the same genotype group (B) as her sister (#I20). Abbreviations: 11OHD, 11-hydroxylase deficiency; 21OHD, 21-hydroxylase deficiency; F, female; M, male; NA, not applicable; NC, not classifiable; U, undefined; yr, year; yrs, years.

Treatment with glucocorticoids	History of salt wasting	History of severe stress while untreated	cortisol before ACTH	cortisol after ACTH
never	yes	hospital admission for vomiting, dengue hemorrhagic fever	61	60
never	no	no	139	129
never	no	no	101	64
never	no	tonsillectomy, dengue hemorrhagic fever	146	149
treatment started after ACTH stimulation test		NA	30	70
treatment started after ACTH stimulation test		NA	30	90

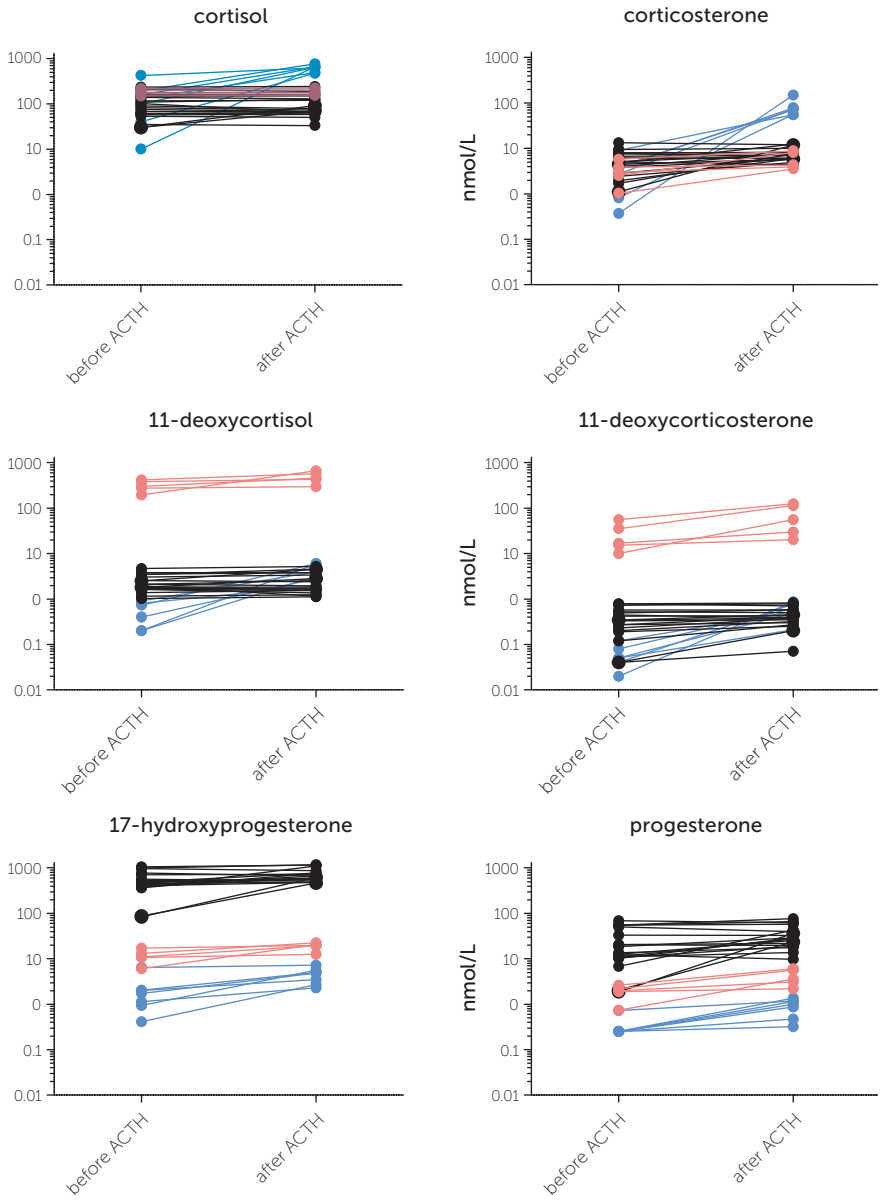


Figure 2 Steroid concentrations in untreated CAH patients.

Blood adrenal steroid concentrations were determined before and 60 minutes after ACTH administration. Black indicates 21-hydroxylase deficient patients, pink indicates 11-hydroxylase deficient patients, and blue indicates control.

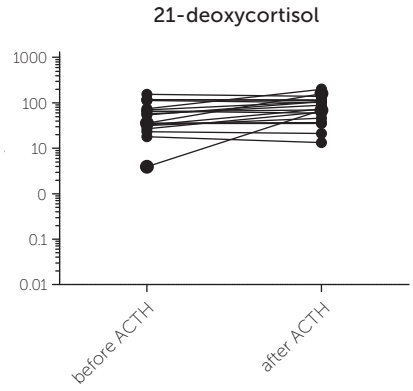


Figure 2 Continued.

Table 2 (Relative) potency of steroids to activate the human glucocorticoid receptor (hGR).

	EC50 (95% CI)	Relative potency to hGR
cortisol	11 (5.9-20) nM	100%
corticosterone	17 (8.9-32) nM	64 %
21DF	22 (12-42) nM	49%
11-hydroxyprogesterone	47 (30-73) nM	23%
11-deoxycortisol	71 (56-92) nM	15%
aldosterone	111 (72-171) nM	9.8%
11-deoxycorticosterone	725 (422-1246) nM	1.5%
progesterone	1147 (664-1981) nM	0.95%
17OHP	1668 (1300-2140) nM	0.65%
16-hydroxyprogesterone	NC *	
pregnenolone	NA	
17OHpregnenolone	NA	

Data of the transactivation assays in human embryonic kidney cells were used to calculate dose-response curves using nonlinear regression. EC50 values could be calculated when a complete dose-response curves was available, including a concentration in which maximum transactivation was reached (plateau phase). * some transactivation at 100,000 nM, but no maximum transactivation was reached with the concentrations tested. Abbreviations: CI, confidence interval; EC50, estimated concentration that causes 50% of the maximum transactivation; hGR, glucocorticoid receptor; NA, not applicable; NC, not calculable.

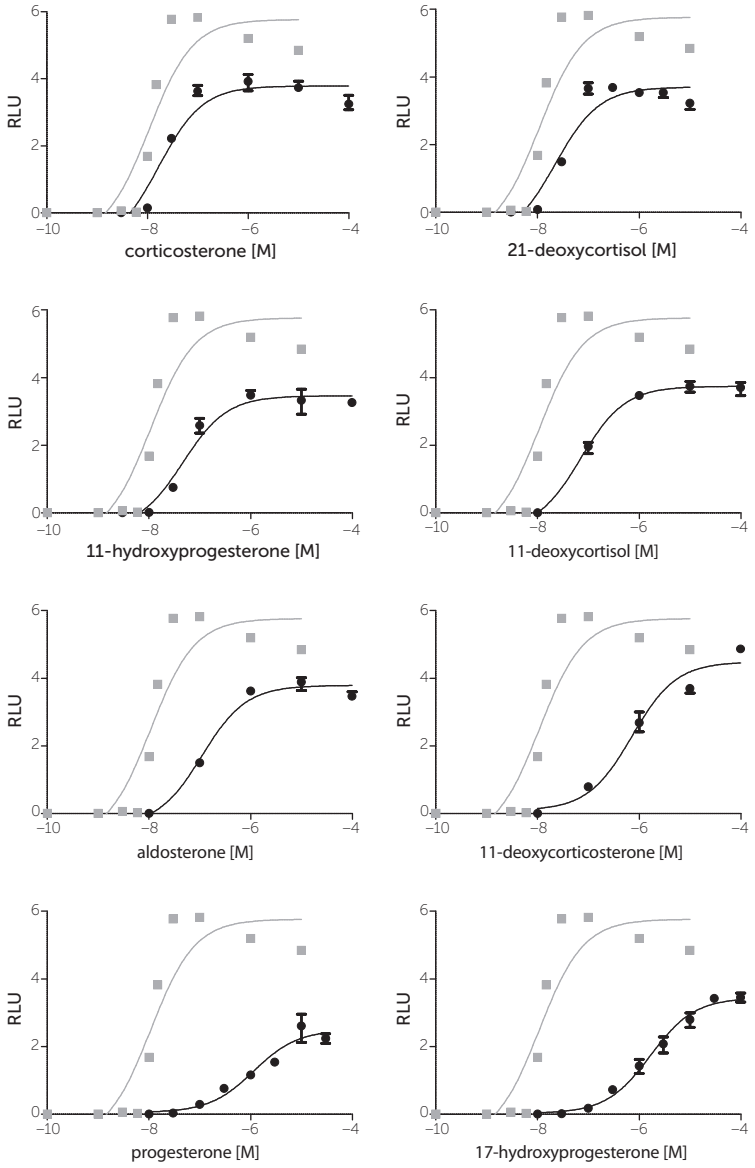


Figure 3 Glucocorticoid receptor transactivation by adrenal steroids.

RLU is a measure for glucocorticoid receptor transactivation as measured by a dual-luciferase assay in human embryonic kidney cells that were exposed to increasing amount of steroids (depicted in black) for 24 hours. Cortisol was used as a reference steroid (depicted in grey). All concentrations were measured in triplicate and mean and range are depicted. Abbreviations: RLU, relative light units.

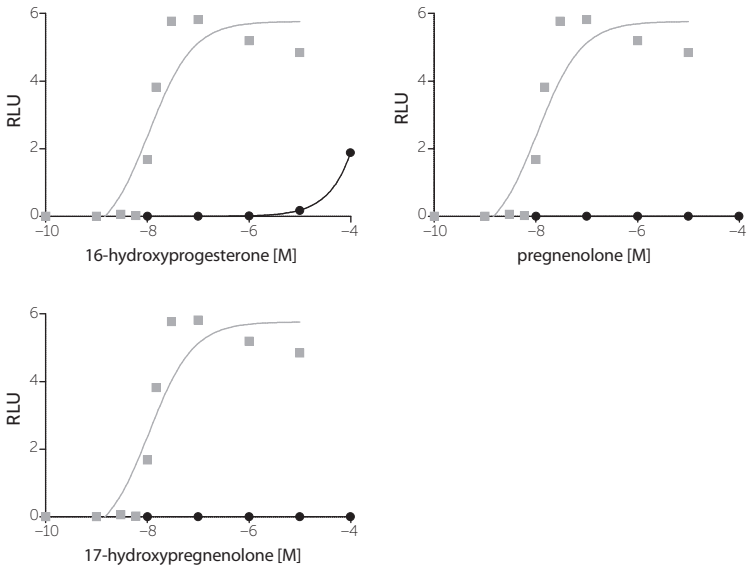


Figure 3 Continued.

Discussion

Here, we describe a unique group of untreated CAH patients with biochemically confirmed severe cortisol deficiency. None of these patients had clinical signs of cortisol deficiency and more than half of these patients report a history of severe stress-situations, such as surgery or severe infectious diseases, and recovered without glucocorticoid stress dosing. We show here that this might be explained by the glucocorticoid activity of significantly increased steroid precursor concentrations, that at least partially compensate the cortisol deficiency.

To the best of our knowledge we are the first to report on measurement of multiple adrenal steroid precursors in an unique cohort of untreated CAH patients. As expected from their enzymatic defect, in untreated 21OHD patients 17OHP (127x higher), progesterone (29x higher), and 21DF (only measurable in 21OHD) concentrations were significantly increased compared to controls. Remarkably, this corresponds to the measured concentrations of adrenal steroid precursors in blood of treated 21OHD patients. [12] Furthermore, in untreated 11OHD patients, we found 11-deoxycortisol (457x higher) and 11-deoxycorticosterone (55x higher) to be the most important accumulating steroid precursors. We hypothesized that these strongly elevated adrenal precursor concentrations may have a stimulating effect on the hGR.

Steroid hormone action is initiated by binding of the hormone to its receptor. The hormone-receptor complex subsequently will translocate to the nucleus were it binds to hormone response elements in the regulatory region of target gene promoters, initiating transactivation. [13] Several studies have been performed evaluating the binding of steroids to the hGR and mineralocorticoid receptor (hMR) and/or the nuclear translocation of the receptor-complex, but only a few studies report on hGR transactivation [8, 14-18], mainly focusing on aldosterone and cortisol, showing that cortisol has higher potency for hGR transactivation than aldosterone. [14, 15, 17, 18] It is nowadays well established that adrenal steroids have steroid hormone cross-reactivity explained by the high degree of homology of the DNA binding domain of the hGR and the hMR. [19] Only one study showed that 21DF, 17OHP and progesterone are able to activate the hGR. [8] To the best of our knowledge, we are the first to describe the potency to activate the hGR for more than 10 adrenal steroid precursors relative to cortisol. We found that 21DF and corticosterone had similar potency to activate the hGR, while the potency of 11-hydroxyprogesterone, 11-deoxycortisol and aldosterone was 4-10x lower, and the potency of 11-deoxycorticosterone, progesterone and 17OHP more than 65x lower compared to cortisol.

Comparison of the transactivation data with the molecular structure of the adrenal steroid precursors shows that dehydrogenation of the steroid precursors by 3β-hydroxysteroid dehydrogenase is a prerequisite to enable a steroid to activate the hGR, as pregnenolone and 17-hydroxypregnenolone, which are not dehydrogenated, were not able to cause hGR transactivation *in vitro*. We also observed that dehydrogenation alone (progesterone) or combined with 21-hydroxylation (11-deoxycorticosterone) or 17-hydroxylation (17OHP) resulted in relatively low potency to activate the hGR. Combination of dehydrogenation and hydroxylation of position 11 and 21 (corticosterone), or position 11 and 17 (21DF), or position 17 and 21 (11-deoxycortisol), increased the potency to activate the hGR as EC50 values were similar or 7 times higher than of cortisol. Strikingly, the most potent hGR activation precursor steroids are 11β-hydroxylated steroids. This confirms the hypothesis of Hellal-Levy et al.[15] and Rousseau et al. [20] that hydroxylation at position 11 enhances glucocorticoid activity.

The strongly increased concentrations of the 11-hydroxylated steroid precursors 21DF and 11-hydroxyprogesterone in untreated 21OHD patients, that are able to activate the hGR, might at least partially compensate for their cortisol deficiency. 17OHP and progesterone might also contribute as we found increased concentrations in 21OHD and 11OHD patients, although the potential to activate the hGR is lower. Furthermore, in 11OHD patients, 11-deoxycortisol and 11-deoxycorticosterone also might compensate for the cortisol deficiency as these steroids had good hGR activation potency.

A clinical threshold of 500 nmol cortisol/L blood after ACTH administration is widely used to define adrenal insufficiency, and although lower thresholds also have been proposed [9-11, 21], all of our patients had cortisol concentrations far below this threshold. Previously, it has been suggested to include serum corticosterone, 11-deoxycorticosterone and 11-deoxycortisol in addition to serum cortisol to evaluate adrenal function. [22]. Based on our finding of glucocorticoid receptor activation we emphasize the importance to use multiple adrenal steroid precursors, such as 11-deoxycortisol, 11-deoxycorticosterone, 11-hydroxyprogesterone, 21DF, 17OHP, progesterone, and cortisol to evaluate glucocorticoid action. More studies are necessary to study the clinical significance of our findings in more detail.

Although our data suggest that CAH patients may survive without glucocorticoid treatment, it has to be pointed out that based on our results we do not advocate to withhold treatment in severely affected CAH patients. However, it has to be noted that in treated CAH patients blood concentrations of the elevated adrenal steroid precursors are generally suppressed and consequently patients become more dependent on glucocorticoid treatment and additional glucocorticoid stress dosing in case of severe illness. As knowledge and infrastructure are far from perfect in developing countries such as Indonesia, treated CAH patients may have an increased risk to develop adrenal crises as the compensatory effect of steroid precursors is absent. Also, acute discontinuation of glucocorticoid treatment, which is likely in developing countries as medication is not always available, can increase the risk to develop adrenal crises as the compensatory steroid precursors will be suppressed for some time. Patients and clinicians should be aware of the importance of daily glucocorticoid supplementation without discontinuation in order to prevent adrenal crises. For the same reason, the importance of an appropriate stress dosing regimen has to be emphasized.

Despite this being the first study in a relatively large cohort of untreated CAH patients, our study has some limitations. In some of the reported stress-situations, there may have been an underlying adrenal crisis as some episodes were reported as salt wasting crises or severe vomiting and/or seizures. Still, it is remarkable that all patients recovered from these episodes without administration of glucocorticoid medication. Furthermore, none of our patients had the most severe type of CAH, a homozygous deletion of *CYP21A2*, although several patients were classified as genotype group 0, with an expected enzyme activity of <1%. As neonatal screening is not implemented in Indonesia, the prevalence of CAH patients is probably higher than the current number of patients diagnosed with CAH, which might have led to selection of our patient group. Especially male CAH patients are expected to be underdiagnosed, either because they have no complaints or because they have died within the first weeks of life due to a salt wasting crisis. Education programs in these countries should therefore also focus on salt requirements in neonates to prevent salt wasting crises.

In conclusion, our results show that severely affected untreated CAH patients with proven cortisol deficiency might survive due to accumulated adrenal steroid precursors which can activate the hGR, and might at least partially compensate for the cortisol deficiency. Especially 21DF, 11-hydroxyprogesterone, 11-deoxycortisol and 17OHP can contribute to this compensation. Therefore, we suggest to measure all relevant hGR activating steroids in the evaluation of adrenal function, especially in CAH patients. Further research should focus on establishing cut-off values for the combined adrenal steroid concentrations and to personalize treatment necessities according to the total panel.

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Supplemental material – LC-MS/MS protocol

Cortisol, 21DF, 11-deoxycortisol, 17OHP, and progesterone were analyzed by LC-MS/MS after protein precipitation and solid-phase extraction. Internal standard $[^2H_4]$-cortisol, $[^2H_4]$-21-deoxycortisol, $[^2H_5]$-11-deoxycortisol, $[^{13}C_3]$-17-hydroxyprogesterone (Isoscience, King of russia, PA) and $[^2H_9]$-progesterone (CDN isotopes) was added to 100 µL serum. Subsequently 300 µL Acetonitrile + 0.1% formic acid was added for protein precipitation. 300 µL H_2O was added to 200 µL supernatant followed by solid phase extraction (Oasis HLB 1cc, Waters). The eluate (methanol/isopropanol 95:5) was dried under a stream of N_2 gas, reconstituted in methanol: water (3:7) and injected (10 µL) into an Agilent Technologies 1290 Infinity UHPLC-system (Agilent Technologies, Santa Clara, CA) equipped with a BEH C18 (1.7µm 2.1 X 50mm) analytical column (Waters Corp.) at 60°C. Mobile phase A (methanol:water 20:80 + 2 mM NH_4CH_3COO + 0.1% formic acid) and B (methanol:water 98:2 + 2 mM NH_4CH_3COO + 0.1% formic acid) were run in a gradient (0.4 mL/min). Start gradient 70:30 A:B for 2.5 min; then to 40:60 A:B in 3.5 min; followed by a gradient in 0.5 min to 2:98 to remain such for 0.5 min and thereafter to 70:30 A:B in 0.5 min and remain such for 0.5 min. Retention time was 1.41 min, 2.13 min, 2.56 min, 4.66 min and 6.04 min for cortisol, 21DF, 11-deoxycortisol, 17OHP and progesterone respectively. Total run time was 8 minutes. An 9-point calibration curve was used (cortisol, 21DF, 11-deoxycortisol, 17OHP (Steraloids), progesterone (Sigma). An Agilent 6490 tandem mass spectrometer (Agilent Technologies) was operated in the electrospray positive ion mode, with a capillary voltage 3.5 kV, fragmentor voltage 380 V, sheath gas temperature 350°C and gas temperature 150 °C with N_2 collision gas. Two transitions (qualitative and quantitative) were monitored. Transitions (Q1>Q3) were m/z 363.4 > 97.1 (34 V) and m/z 363.4 > 121.1 (25 V) for cortisol; m/z 367.4 > 97.1 (34 V) and m/z 367.4 > 121.1 (25 V) for $[^2H_4]$-cortisol. m/z 347.2 > 121.1 (27 V) and m/z 347.2 > 269.0 (18 V) for 21DF; m/z 351.2 > 121.0 (29 V) and m/z 351.2 > 273.0 (18 V) for $[^2H_4]$-21-deoxycortisol. m/z 347.2 > 109.1 (31 V) and m/z 347.2 > 97.1 (29 V) for 11-deoxycortisol; m/z 352.3 > 113.1 (29 V) and m/z 352.3 > 100.1 (31 V) for $[^2H_5]$-11-deoxycortisol. m/z 331.3 > 109.1 (31 V) and m/z 331.3 > 97.1 (31 V) for 17OHP; m/z 334.3 > 112.1 (33 V) and m/z 334.3 > 100.1 (30 V) for $[^{13}C_3]$-17-hydroxyprogesterone. m/z 315.3 > 109.1 (29 V) and m/z 315.3 > 97.1 (29 V) for progesterone; m/z 324.3 > 113.1 (29 V) and m/z 324.3 > 100.1 (29 V) for $[^2H_9]$-progesterone. Dwell time was 100 ms, 20 ms, 20 ms, 60 ms and 100 ms for cortisol, 21DF, 11-deoxycortisol, 17OHP, and progesterone respectively. The method was linear assessed by CLSI EP6 protocol. For cortisol total CV is 3.6% at 300 nmol/l and 3.1% at 1080 nmol/l. For 21DF total CV is 9.6% at 1.1 nmol/l and 8.6% at 15.2 nmol/l. For 11-deoxycortisol total CV is 5.9% at 2.1 nmol/l and 5.2% at 26.9 nmol/l. For 17OHP total CV is 5.6% at 2.6 nmol/l and 5.1% at 94.3 nmol/l. For progesterone total CV is 5.9% at 5.1 nmol/l and 3.9% at 30.4 nmol/l.

Corticosterone and 11-deoxycorticosterone were analyzed by LC-MS/MS after protein precipitation and solid-phase extraction. Internal standard [$^{2}H_4$]-corticosterone and [$^{13}C_3$]-11-deoxycorticosterone (Isoscience, King of Prussia, PA) was added to 100 µL serum. Subsequently 300 µL Acetonitrile + 0.1% formic acid was added for protein precipitation. 500 µL H_2O was added to 200 µL supernatant followed by solid phase extraction (Oasis HLB 1cc, Waters). The eluate (methanol/water 90:10) was dried under a stream of N_2 gas, reconstituted in methanol: water (3:7) and injected (10 µL) into an Agilent Technologies 1290 Infinity UHPLC-system (Agilent Technologies, Santa Clara, CA) equipped with a HSS T3 (1.8µm 2.1 X 100mm) analytical column (Waters Corp.) at 40°C. Mobile phase A (methanol:water 20:80 + 2 mM NH_4CH_3COO + 0.1% formic acid) and B (methanol:water 98:2 + 2 mM NH_4CH_3COO + 0.1% formic acid) were run in a gradient (0.4 mL/min). Start gradient 60:40 A:B for 3.5 min; then to 48:52 A:B in 1 min and 38:62 A:B in 3 min; followed by a gradient in 0.01 min to 5:95 A:B to remain such for 1 min and thereafter to 60:40 A:B in 0.5 min and remain such for 2 min. Retention time was 5.6 min and 7.2 min for corticosterone and 11-deoxycorticosterone, respectively. Total run time was 11 minutes. A 9-point calibration curve was used (corticosterone and 11-deoxycorticosterone (Sigma)). An Agilent 6490 tandem mass spectrometer (Agilent Technologies) was operated in the electrospray positive ion mode, with a capillary voltage 3.0 kV, fragmentor voltage 380 V, sheath gas temperature 350°C and gas temperature 150 °C with N_2 collision gas. Two transitions (qualitative and quantitative) were monitored. Transitions (Q1>Q3) were m/z 347.2 > 97.0 (31 V) and m/z 347.2 > 121.1 (29 V) for corticosterone; m/z 351.3 > 97.1 (31 V) and m/z 351.3 > 121.1 (29 V) for [$^{2}H_4$]-corticosterone; m/z 331.3 > 109.1 (31 V) and m/z 331.3 > 97.1 (29 V) for 11-deoxycorticosterone; m/z 334.3 > 112.2 (29 V) and m/z 334.3 > 100.2 (27 V) for [$^{13}C_3$]-11-deoxycorticosterone. Dwell time was 100 ms for both corticosterone and 11-deoxycorticosterone. The method was linear assessed by CLSI EP6 protocol. For corticosterone total CV is 3.2% at 26.8 nmol/l and 3.6% at 59.4 nmol/l. For 11-deoxycorticosterone total CV is 3.7% at 0.2 nmol/l and 2.7% at 2.0 nmol/l.

Chapter 8

Influence of 17-hydroxyprogesterone, progesterone and sex steroids on mineralocorticoid receptor transactivation in congenital adrenal hyperplasia

Mooij CF, Parajes S, Pijnenburg-Kleizen KJ, Arlt W, Krone N, Claahsen-van der Grinten HL. Horm res paediatr. 2015; 83: 414-21

Abstract

Background Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency leads to accumulation of steroid precursors and adrenal androgens. These steroids may have a biological effect on the steroid receptor with clinical consequences on diagnostics and treatment in CAH patients. Therefore, we analysed the effect of accumulated steroids [17-hydroxyprogesterone (17OHP), progesterone, androstenedione and testosterone] on aldosterone-mediated transactivation of the human mineralocorticoid receptor (hMR).

Methods A transactivation assay using transiently transfected COS7 cells was employed. Cells were co-transfected with hMR-cDNA, MMTV-luciferase and renilla-luciferase expression vectors. Transfected cells were incubated with six different steroid concentrations in addition to aldosterone (10^{-10}M). Luciferase and renilla activities were measured to quantify hMR transactivation.

Results Linear regression analysis showed statistically significant linear inhibition of transactivation of the hMR by 10^{-10}M aldosterone in the presence of increasing 17OHP [$F(1,5) = 11.34$, $p = 0.019$] and progesterone [$F(1,5) = 11.08$, $p = 0.021$] concentrations. In contrast, neither androstenedione nor testosterone affected hMR transactivation by aldosterone at a concentration of 10^{-10}M.

Conclusions Our study shows for the first time that neither androstenedione nor testosterone has a biological effect on aldosterone-mediated transactivation of the hMR. 17OHP and progesterone have an anti-mineralocorticoid effect in vitro that may clinically lead to an increased requirement of mineralocorticoids in poorly controlled CAH patients.

Introduction

Congenital adrenal hyperplasia (CAH) is a group of disorders affecting adrenal steroidogenesis. The incidence of classic CAH varies between 1 in 10,000 and 1 in 15,000 live births in most Caucasian populations. [1] In about 95% of the cases, CAH is caused by 21-hydroxylase deficiency [2], resulting in impaired adrenal synthesis of cortisol. Cortisol deficiency triggers a counter-regulatory increase in pituitary ACTH secretion leading to accumulation of adrenal steroid precursors before the deficient enzymatic step and increased adrenal androgen production. 21-hydroxylase converts 17-hydroxyprogesterone (17OHP) to 11-deoxycortisol, the penultimate step in cortisol synthesis. Hence, 17OHP accumulates and is used as a marker for 21-hydroxylase deficiency.

Classic CAH is commonly subdivided in the salt wasting (SW) and simple virilizing (SV) forms depending on the residual enzymatic activity. SW patients have no residual 21-hydroxylase activity leading to severe salt loss, typically after the first week of life, and prenatal virilization of the female external genitalia. Patients with the SV form of CAH have a residual enzyme activity of 1-2% and usually have sufficient aldosterone production to prevent severe salt loss, whereas glucocorticoid synthesis is severely impaired. In both SW and SV forms, elevated adrenal androgens cause prenatal virilization of the female external genitalia and postnatal androgen excess in both sexes. [2,3] Current treatment of CAH consists of lifelong glucocorticoid and, if necessary, mineralocorticoid treatment. [4] Treatment with glucocorticoids restores feedback within the hypothalamus-pituitary-adrenal axis, consequently achieving downregulation of adrenal androgen production. In many patients, however, supraphysiological doses of glucocorticoids are needed to normalize androgen levels.

Untreated and poorly controlled CAH patients are characterized by elevated levels of steroid hormone precursors, including progesterone and 17OHP, and androgens such as androstenedione and testosterone. [3,5,6,7,8] It has been shown that progesterone and 17OHP have antagonistic properties on the human mineralocorticoid receptor (hMR), and therefore may contribute to mineralocorticoid deficiency in classic CAH patients. [9] The aim of our study was to evaluate the effects of 17OHP, progesterone, androstenedione and testosterone on aldosterone-mediated transactivation and translocalization of the hMR. Furthermore, we studied the effect of the frequent mineralocorticoid receptor p.Ile180Val single nucleotide polymorphism (SNP) on transactivation of the hMR.

Material and methods

Construction of plasmids

The hMR cDNA was PCR amplified from the previously used pcDNA3.1-*NR3C2* construct [10] using specific primers with *Hind*III and *Eco*RV restriction sites for directional cloning into pcDNA6/V5-His-B vector (Invitrogen Corp., Carlsbad, Calif., USA). The p.Ile180Val SNP was recreated in the pcDNA6-hMR construct by site-directed mutagenesis using the QuikChange XL Site-Directed Mutagenesis Kit according to the manufacturer's protocol (Stratagene, Amsterdam, The Netherlands). The correct insertion of the hMR construct and the p.Ile180Val SNP as well as the integrity of the cDNA was checked by direct DNA sequencing. For intracellular localization assays, Green Fluorescent Protein (GFP), an autofluorescent genetic reporter, was cloned into pcDNA6. The hMR cDNA and the hMR p.Ile180Val (hMR-I180V) construct were cloned into the pcDNA6-GFP vector using the same restriction enzymes as described above.

In vitro transactivation assays

Transactivation of hMR and hMR-I180V by different concentrations of aldosterone was investigated using a MMTV-luciferase assay. Approximately 2.5×10^4 COS-7 cells were grown in 500 ml of Dulbecco's minimal essential medium (DMEM) high glucose (4.5 g/l) with L-glutamine (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH) and penicillin/streptomycin (PAA Laboratories GmbH) in 24-well plates, and transiently transfected 24 h after seeding using FuGene® HD transfection reagent (Roche Applied Sciences, Burgess Hill, UK). Cells were transfected with 300 ng of pcDNA6-V5/HisB-hMR or pcDNA6-V5/HisB-hMR variant (p.Ile180Val) in the presence of 300 ng of a mouse mammary tumour virus (MMTV)-luciferase reporter construct (MMTV-luc) driving the firefly luciferase gene. Co-transfection with 50 ng of pRL-TK (Promega, Madison, Wisc., USA), a renilla luciferase vector, was performed to normalize data for transfection efficiency. In each set of experiments, 3 wells with COS-7 cells were co-transfected with 300 ng of pcDNA-hMR and 300 ng of pGL3-Basic (Promega) for data normalization and interassay comparison purposes as pGL3-Basic contains a coding region for firefly luciferase for monitoring transcriptional activity in transfected cells. Two days after transfection, cells were treated with aldosterone (Sigma Aldrich, Gillingham, UK) for 24 h in different concentrations (final concentrations made up in total of 500 µl full DMEM media: 10^{-6}, 10^{-8}, 10^{-10}, 10^{-12} and 10^{-14}M), or in a 10^{-10}M concentration in addition to different concentrations of 17OHP (range 5-1,000 nM), progesterone (2.5-100 nM), androstenedione (1-250 nM) or testosterone (0.5-60 nM) (Sigma Aldrich). Concentrations of 17OHP, progesterone, androstenedione and testosterone used in the assays were based on biochemical findings in CAH patients [5,6,7,8].

To evaluate the transactivational potential of 17OHP, progesterone, androstenedione and testosterone on the hMR in the absence of aldosterone, transfected cells were also incubated in 500 µl of full DMEM supplemented with different concentrations of these steroids.

Cells were lysed in 100 µl of passive lysis buffer (Promega). Consequently, 30 µl of cell lysate was used for the measurement of firefly and renilla luciferase activity, with a luminometer (Berthold, Bad Wildbad, Germany), using the Dual-Luciferase® Reporter Assay System (Promega) according to manufacturer's standard protocol. The hMR transactivation was calculated by the ratio of the steroid-dependent (firefly) luciferase and the steroid independent renilla (luciferase). Luciferase/renilla ratios were normalized for luciferase activity driven by pGL3-Basic. Data were normalized for the transactivation by a 10^{-10}M aldosterone concentration and are presented as fold transactivation compared to the transactivation by 10^{-10}M aldosterone (transactivation by 10^{-10}M aldosterone was set as 1.0-fold transactivation). All assays were performed in triplicate (n = 9). Statistical analysis was performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, Calif., USA). Results were analysed by both linear regression analyses and ANOVA with Bonferroni adjustment for multiple comparisons (all possible comparisons were analysed). Differences between the hMR wild type and the p.Ile180Val construct were analysed using a t-test. $p < 0.05$ was considered significant.

Intracellular localization

The transactivation potential of the hMR-GFP construct was evaluated to ensure comparable transactivation potential to the hMR construct in the presence of 10^{-10}M concentrations of aldosterone. The hMR-GFP construct was used for an intracellular localization assay. Approximately 2×10^5 COS-7 cells were grown on glass coverslips in 6-well plates containing 2 ml of DMEM high glucose (4.5 g/l) with L-glutamine (PAA Laboratories GmbH) supplemented with charcoal stripped fetal bovine serum (Sigma Aldrich) and penicillin/streptomycin (PAA Laboratories GmbH). Twenty-four hours after seeding, the cells were transiently transfected using FuGene® HD transfection reagent (Roche Applied Sciences) with 2 µg of hMR-GFP or 2 µg of hMR-I180V-GFP. Forty-eight hours after transfection, cells were treated for 120 min with a combination of 10^{-10}M aldosterone and different concentrations of other steroids (17OHP, progesterone, androstenedione and testosterone) to study the effect of these steroids on the intracellular localization of the receptor. Cells were washed three times in 1x phosphate buffered saline (PBS) and fixed in 1 ml 100% methanol at -20°C for 15 min. Fixed cells were further washed three more times in 1x PBS and mounted on Vectorshield with 4', 6-diamidino-2-phenylindole (DAPI; exclusively nuclear staining). Results were obtained from three independent transfection experiments in which 150 transfected cells were classified in 4 categories:

(1) nuclear, (2) mainly nuclear, (3) equal nuclear and cytoplasmic, and (4) mainly cytoplasmic. Representative images were taken using confocal microscopy (Nikon Instruments Inc., Melville, N.Y., USA). To evaluate if treatment causes a difference in the number of cells counted as nuclear, mainly nuclear, equal nuclear or mainly cytoplasmic, respectively, one-way ANOVA was performed. Statistical analysis was performed using GraphPad Prism software version 5.0.

Results

Transactivation of the mineralocorticoid receptor by aldosterone

Increasing concentrations of aldosterone caused an increase in potent transactivation of both the hMR and hMR-I180V. The dose-dependent effects on the transactivation are shown in a dose-response curve (Figure 1). An estimated concentration for 50% transactivation (EC50) of the hMR of around 10^{-10}M aldosterone was calculated for both the wild type (2.4×10^{-11}M) and the p.Ile180Val SNP (1.2×10^{-11}M).

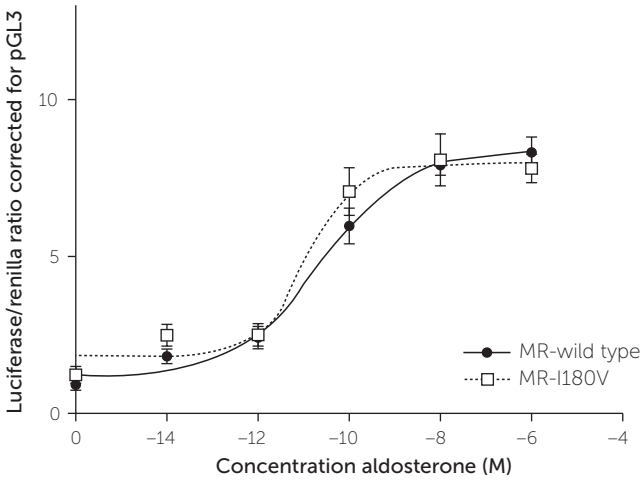

Figure 1 Dose-response curves showing the transactivation of the hMR (wild type) and the hMR-I180V SNP by different concentrations of aldosterone using a luciferase assay.

The results are expressed as the ratio of (firefly) luciferase and renilla (luciferase) activity. Data are means ± SEM for each concentration (n = 9). MR = Mineralocorticoid receptor.

Effect of 17OHP, progesterone, androstenedione and testosterone on hMR transactivation

Increasing concentrations of 17OHP and progesterone inhibited aldosterone-mediated transactivation of the hMR in a dose-dependent fashion (Figure 2). Linear regression analyses showed a linear inhibition of transactivation of the hMR by 10^{-10}M aldosterone in the presence of increasing concentrations of 17OHP [$F(1,5) = 11.34$, $p = 0.019$] and progesterone [$F(1,5) = 11.08$, $p = 0.021$]. Variable concentrations of 17OHP [$F(6,48) = 111.9$, $p < 0.0001$] and progesterone [$F(6,48) = 62.11$, $p < 0.0001$] have a significant effect on transactivation of the hMR by aldosterone in the presence of 10^{-10}M aldosterone, as shown by ANOVA (Supplemental Tables 1, 2).

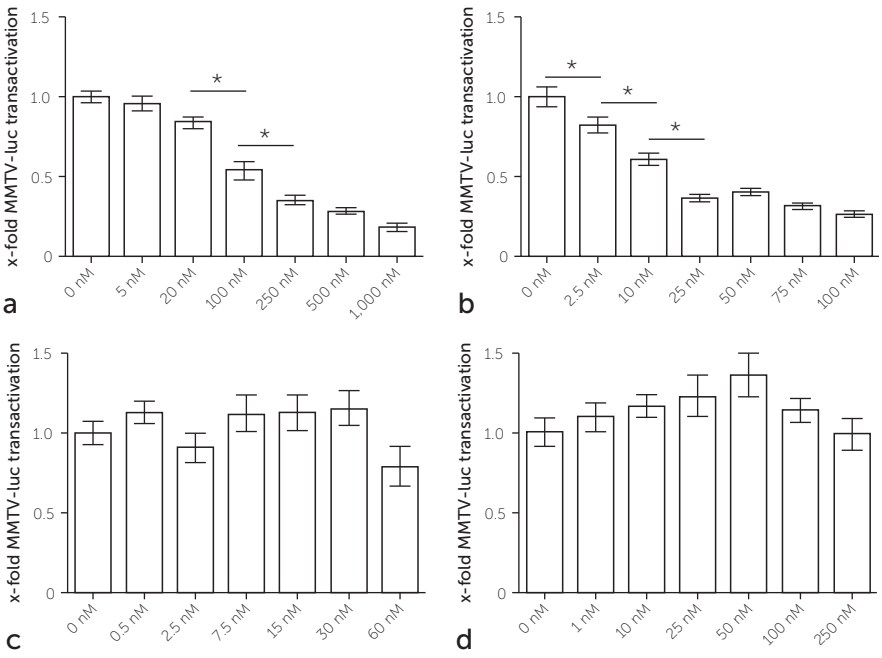

Figure 2 The effect of different concentrations of 17OHP (a), progesterone (b), testosterone (c) and androstenedione (d) on the transactivation of hMR by 10^{-10}M aldosterone concentration.

The transactivation activity of 10^{-10}M aldosterone was set as 1.0. Results are expressed as x-fold transactivation of MMTV (firefly) luciferase (MMTV-luc). Data are means ± SEM for each concentration (n = 9). Significant differences in transactivation between two concentrations closest to each other are indicated by an asterisk ($p < 0.05$).

In contrast, treatment with increasing concentrations of androstenedione and testosterone did not have any measureable effect on hMR transactivation (Figure 2). No linear effect of increasing concentrations of androstenedione [F(1,5) = 0.709, p = 0.438] or testosterone [F(1,5) = 1.57, p = 0.265]on transactivation of the hMR by aldosterone was found. In addition, ANOVA showed that different concentrations of androstenedione or testosterone did not affect transactivation of the hMR by aldosterone (Supplemental Tables 3, 4).

The effect of three different concentrations of 17OHP on the aldosterone-mediated transactivation of the hMR was also evaluated in the p.Ile180Val SNP construct (Figure 3). The inhibitory effect of 17OHP on hMR-I180V was found to be similar to its effect on the wild-type hMR (p > 0.05).

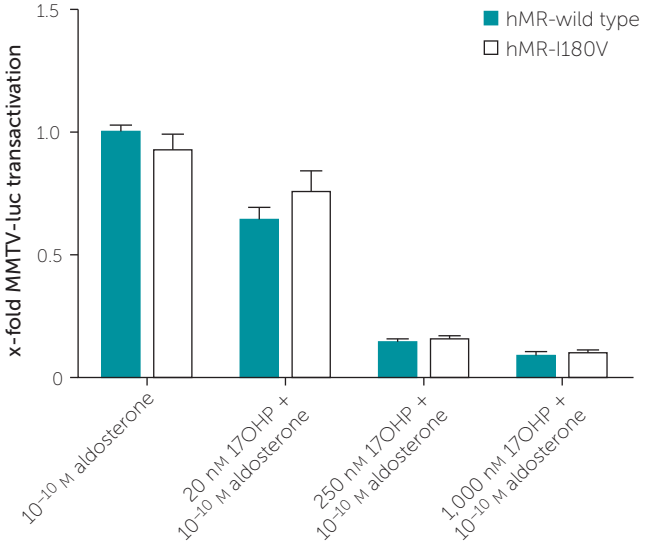

Figure 3 The effect of different concentrations of 17OHP on the transactivation of hMR by 10^{-10}M aldosterone concentration compared to the effect of different concentrations of 17OHP on the transactivation of the hMR-I180V SNP.

The transactivation activity of 10^{-10}M aldosterone on the hMR (wild type) was set as 1.0. Results are expressed as x-fold transactivation of MMTV (firefly) luciferase (MMTV-luc). Data are means ± SEM for each concentration (n = 9).

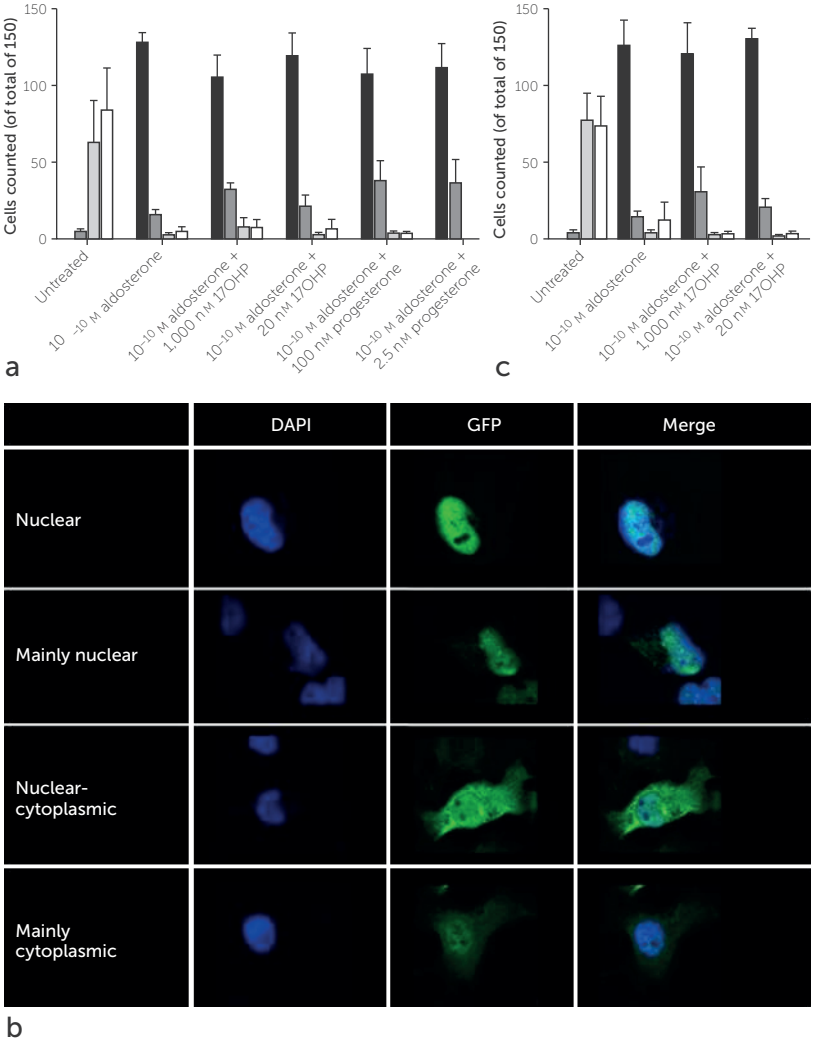

Figure 4 a Cellular localization of the hMR without the presence of aldosterone and in the presence of aldosterone with or without different concentrations of 17OHP and progesterone.

Cells were localized using confocal microscopy as (1) nuclear (black bars), (2) mainly nuclear (dark grey bars), (3) equal nuclear-cytoplasmic (light grey bars) and (4) mainly cytoplasmic (white bars). b Images showing the four possible cellular localizations of the hMR: (1) nuclear, (2) mainly nuclear, (3) equal nuclear and cytoplasmic, (4) mainly cytoplasmic. Images were taken with a confocal microscope. Different images were taken showing DAPI staining, GFP and a merged image. c Cellular localization of the hMR-I180V without the presence of steroids and in the presence of aldosterone with or without different concentrations of 17OHP. Cells were localized using confocal microscopy as (1) nuclear (black bars), (2) mainly nuclear (dark grey bars), (3) equal nuclear - cytoplasmic (light grey bars) and (4) mainly cytoplasmic (white bars).

Intracellular localization of the hMR

The transactivation potential of both the hMR-GFP and the hMR construct were compared to assess whether the GFP had altered transactivation properties of the construct prior to performing an intracellular localization assay. The hMR-GFP construct was shown to have equal transactivational properties as the hMR construct (Supplemental Figure 1).

In untreated cells, the hMR was localized only in the cytoplasm or equally distributed in nucleus and cytoplasm (Figure 4a). Treatment with aldosterone for 120 min resulted in a clear translocation of the hMR with a predominantly nuclear localization.

17OHP and progesterone did not influence the translocation of the hMR to the nucleus in the presence of aldosterone (Figure 4b). Treatment with 17OHP, progesterone, androstenedione or testosterone did not result in significant differences in the intracellular localization of the hMR.

In the presence of aldosterone, the hMR-I180V-GFP was also mainly localized in the nucleus. 17OHP did not inhibit the translocation of the hMR-I180V-GFP to the nucleus in the presence of aldosterone (Figure 4c).

Discussion

We studied the effects of different adrenal steroid hormone precursors and androgens on the transactivation potential and localization of the hMR. Our study shows for the first time that excess concentrations of androstenedione and testosterone do not have a biological effect on the aldosterone-mediated transactivation of the hMR *in vitro*. Furthermore, 17OHP and progesterone have a strong anti-mineralocorticoid effect *in vitro*, which confirms previous findings. [9] This study highlights the anti-mineralocorticoid effect of elevated 17OHP concentrations as found in poorly controlled CAH patients.

These findings may have important implications for clinical care. Based on our results, it can be suggested that elevated 17OHP and progesterone concentrations are likely to have an adverse effect on the mineralocorticoid effect in untreated and poorly treated CAH. This may potentially lead to increased requirement of mineralocorticoids and suboptimal control. In contrast, elevated androgens did not influence the mineralocorticoid transactivation *in vitro*. We therefore hypothesize that elevated androgens per se do not have a clinically relevant effect on mineralocorticoid treatment of CAH.

The current treatment strategy is based on normalizing adrenal androgens to prevent adverse effects of hyperandrogenism. Slightly elevated 17OHP concentrations are generally accepted because of the possible side effects of high dosages of

glucocorticoids needed to achieve physiological 17OHP concentrations. Based on our results it can be suggested that lowering of highly elevated 17OHP concentrations may also have an additional positive effect on the dose of mineralocorticoid treatment and consequently decrease the potential risk of adverse effects of mineralocorticoid treatment such as hypertension. Unfortunately, supraphysiological doses of glucocorticoids are generally necessary to lower 17OHP levels, that may lead to adverse effects and long-term complications. Therefore, the treatment goal in CAH patients is normalization of adrenal androgens with slightly elevated 17OHP levels [4]. Elevated renin levels may indicate the need for higher mineralocorticoid doses. However, based on our data, elevated renin concentrations may also reflect the anti-mineralocorticoid effect of elevated 17OHP concentrations. A fine balance between the use of supraphysiological dosages of glucocorticoids, mineralocorticoid treatment and normalizing 17OHP levels has to be achieved to prevent long-term complications of overtreatment with glucocorticoids on one hand and overtreatment with mineralocorticoids on the other hand.

The antagonistic properties of progesterone on the human, rat and sheep mineralocorticoid receptor have been previously described. [9,11,12,13,14,15] 50% inhibition of the maximum transactivation of the mineralocorticoid receptor is caused by progesterone concentrations between 2 and 11 nM. [9,16,17,18] The inhibitory effect of progesterone described in our study is in line with those described in the studies mentioned above. Minor differences between the results of those studies may be explained by different cells and different luciferase constructs used.

The effect of slightly elevated 17OHP concentrations on the hMR have been studied previously. [9] The previously reported concentration of 135 nM, causing a 50% inhibition of transactivation of the hMR by a 10^{-9}M aldosterone, is in line with the antagonistic effect of 17OHP on aldosterone-mediated transactivation described in our study. In our study we evaluated the effect of even higher 17OHP concentrations, as found in untreated or poorly controlled CAH patients.

In contrast to the effect on transactivation, the translocation to the nucleus seems not to be affected by 17OHP or progesterone. The physiological human ligand of the hMR is aldosterone. After binding to aldosterone, the hMR undergoes a conformational change and partial dissociation of the ligand binding complex occurs, leading to translocation of the hMR to the nucleus. Within the nucleus, the activated receptors regulate transcription by different pathways including transactivation of target genes. [19,20,21,22,23] Intracellular localization studies on the hMR have shown that in the absence of steroids the hMR is localized in the cytoplasm and in the nucleus, while aldosterone causes a rapid nuclear accumulation of the hMR. [19,24,25,26,27] Binding of aldosterone to the hMR causes dissociation of several associated proteins from the receptor, followed by

dimerization and finally nuclear translocation of the activated receptor. The translocation assay performed in this study showed a similar subcellular localization with a predominant localization of the hMR in the cytoplasm in the absence of steroids. Treatment of the COS-7 cells expressing the hMR-GFP construct with aldosterone causes a quick translocation of the hMR to the nucleus of the cells. However, different concentrations of 17OHP and progesterone in addition to an aldosterone concentration of 10^{-10}M do not have an impact on the translocation of the hMR to the nucleus. This finding is in contrast to the described effects of hMR antagonists, such as spironolactone and eplerenone, which inhibit the translocation of the hMR to the nucleus. [19]

The mechanism of the inhibition of aldosterone-mediated transactivation of the hMR by progesterone and 17OHP remains unclear. It has been shown that 17OHP has a relatively high binding affinity for the hMR. [9] Therefore, competitive binding of the hMR between 17OHP and aldosterone, such as in patients with poorly controlled CAH, is very likely. We showed that 17OHP does not inhibit the translocation of the hMR to the nucleus. Therefore, we hypothesize that the anti-mineralocorticoid effect of 17OHP on the hMR is not due to an effect on the translocation of the hMR, but might be caused by effects on the transcription after translocation to the nucleus. It has been suggested by Hellal-Levy et al. [20] that binding of an antagonist to the hMR leads to an inactive conformation of the hMR. Due to instability, this complex of the mineralocorticoid receptor and its antagonist will not be converted into a transcriptionally active conformation. [20] This hypothesis may explain the antagonistic properties of 17OHP and progesterone on the hMR.

The mineralocorticoid receptor p.Ile180Val SNP (rs5522) is one of the most frequent SNPs in the hMR with a frequency of 10.2% of the G allele in a European population (HapMap project, www.hapmap.org). The mineralocorticoid receptor p.Ile180Val SNP has been associated with an increased hypertension risk [28]. As CAH patients have a tendency to develop elevated blood pressure [29,30], the role of this SNP in CAH patients might be important with respect to their cardiovascular risk profile. We showed that the hMR p.Ile180Val SNP does not affect transactivation of the hMR by aldosterone. These findings are in line with the results by DeRijk et al. [31] In addition, 17OHP has the same antagonistic effect on the hMR-I180V SNP as on the wild-type hMR. Thus, the results of this study do not explain the increased hypertension risk in p.Ile180Val.

In conclusion, our study shows for the first time that neither androstenedione nor testosterone have a significant biological effect on the aldosterone-mediated transactivation of the hMR. In contrast, increased 17OHP and progesterone concentrations have an anti-mineralocorticoid effect due to an inhibition of aldosterone-mediated transactivation of the hMR. However, unlike hMR blockers,

neither 17OHP nor progesterone inhibits the translocation of the hMR to the nucleus. Further studies are needed to explain the mechanism of this inhibition of transactivation by 17OHP and progesterone.

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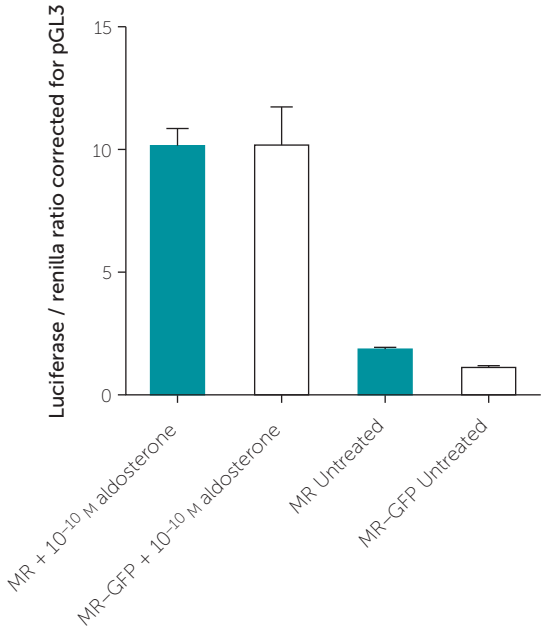
Supplementary material



Supplementary Figure 1 Transactivation potential of the hMR construct versus the hMR-GFP construct evaluated by a luciferase assay.

The results are expressed as the ratio of (firefly) luciferase to renilla (luciferase) activity corrected for pGL3 (transfection efficiency). Data are means ± S.E.M. (n=9).

Table 1 Results of Bonferroni's Multiple Comparison Test for all comparisons in the experiment evaluating the effect of different concentrations of 17OHP on the aldosterone mediated transactivation of the hMR

Comparison	Mean difference.	t	P < 0.05?
1000 nmol/l vs 500 nmol/l	-0,09818	2,176	No
1000 nmol/l vs 250 nmol/l	-0,1691	3,748	Yes
1000 nmol/l vs 100 nmol/l	-0,3538	7,841	Yes
1000 nmol/l vs 20 nmol/l	-0,6562	14,54	Yes
1000 nmol/l vs 5 nmol/l	-0,7723	17,12	Yes
1000 nmol/l vs 0 nmol/l	-0,8160	18,09	Yes
500 nmol/l vs 250 nmol/l	-0,07093	1,572	No
500 nmol/l vs 100 nmol/l	-0,2556	5,664	Yes
500 nmol/l vs 20 nmol/l	-0,5581	12,37	Yes
500 nmol/l vs 5 nmol/l	-0,6741	14,94	Yes
500 nmol/l vs 0 nmol/l	-0,7179	15,91	Yes
250 nmol/l vs 100 nmol/l	-0,1846	4,092	Yes
250 nmol/l vs 20 nmol/l	-0,4871	10,80	Yes
250 nmol/l vs 5 nmol/l	-0,6032	13,37	Yes
250 nmol/l vs 0 nmol/l	-0,6469	14,34	Yes
100 nmol/l vs 20 nmol/l	-0,3025	6,704	Yes
100 nmol/l vs 5 nmol/l	-0,4186	9,277	Yes
100 nmol/l vs 0 nmol/l	-0,4623	10,25	Yes
20 nmol/l vs 5 nmol/l	-0,1161	2,573	No
20 nmol/l vs 0 nmol/l	-0,1598	3,542	Yes
5 nmol/l vs 0 nmol/l	-0,04373	0,9692	No

Table 2 Results of Bonferroni's Multiple Comparison Test for all comparisons in the experiment evaluating the effect of different concentrations of progesterone on the aldosterone mediated transactivation of the hMR.

Comparison	Mean difference	t	P < 0.05?
100 nmol/l vs 75 nmol/l	-0,04790	0,9424	No
100 nmol/l vs 50 nmol/l	-0,1382	2,719	No
100 nmol/l vs 25 nmol/l	-0,09753	1,919	No
100 nmol/l vs 10 nmol/l	-0,3455	6,797	Yes
100 nmol/l vs 2.5 nmol/l	-0,5654	11,12	Yes
100 nmol/l vs 0 nmol/l	-0,7384	14,53	Yes
75 nmol/l vs 50 nmol/l	-0,09030	1,776	No
75 nmol/l vs 25 nmol/l	-0,04963	0,9763	No
75 nmol/l vs 10 nmol/l	-0,2976	5,855	Yes
75 nmol/l vs 2.5 nmol/l	-0,5175	10,18	Yes
75 nmol/l vs 0 nmol/l	-0,6905	13,58	Yes
50 nmol/l vs 25 nmol/l	0,04067	0,8001	No
50 nmol/l vs 10 nmol/l	-0,2073	4,078	Yes
50 nmol/l vs 2.5 nmol/l	-0,4272	8,405	Yes
50 nmol/l vs 0 nmol/l	-0,6002	11,81	Yes
25 nmol/l vs 10 nmol/l	-0,2480	4,878	Yes
25 nmol/l vs 2.5 nmol/l	-0,4679	9,205	Yes
25 nmol/l vs 0 nmol/l	-0,6408	12,61	Yes
10 nmol/l vs 2.5 nmol/l	-0,2199	4,327	Yes
10 nmol/l vs 0 nmol/l	-0,3929	7,729	Yes
2.5 nmol/l vs 0 nmol/l	-0,1729	3,402	Yes

Table 3 Results of Bonferroni's Multiple Comparison Test for all comparisons in the experiment evaluating the effect of different concentrations of testosterone on the aldosterone mediated transactivation of the hMR.

Comparison	Mean difference	t	P < 0.05?
60 nmol/l vs 30 nmol/l	-0,3613	2,501	No
60 nmol/l vs 15 nmol/l	-0,3361	2,326	No
60 nmol/l vs 7.5 nmol/l	-0,3316	2,295	No
60 nmol/l vs 2.5 nmol/l	-0,1137	0,7870	No
60 nmol/l vs 0.5 nmol/l	-0,3324	2,301	No
60 nmol/l vs 0 nmol/l	-0,2067	1,431	No
30 nmol/l vs 15 nmol/l	0,02527	0,1749	No
30 nmol/l vs 7.5 nmol/l	0,02973	0,2058	No
30 nmol/l vs 2.5 nmol/l	0,2476	1,714	No
30 nmol/l vs 0.5 nmol/l	0,02890	0,2001	No
30 nmol/l vs 0 nmol/l	0,1546	1,070	No
15 nmol/l vs 7.5 nmol/l	0,004459	0,03086	No
15 nmol/l vs 2.5 nmol/l	0,2224	1,539	No
15 nmol/l vs 0.5 nmol/l	0,003627	0,02511	No
15 nmol/l vs 0 nmol/l	0,1294	0,8955	No
7.5 nmol/l vs 2.5 nmol/l	0,2179	1,508	No
7.5 nmol/l vs 0.5 nmol/l	-0,0008315	0,005755	No
7.5 nmol/l vs 0 nmol/l	0,1249	0,8646	No
2.5 nmol/l vs 0.5 nmol/l	-0,2187	1,514	No
2.5 nmol/l vs 0 nmol/l	-0,09299	0,6436	No
0.5 nmol/l vs 0 nmol/l	0,1257	0,8704	No

Table 4 Results of Bonferroni's Multiple Comparison Test for all comparisons in the experiment evaluating the effect of different concentrations of androstenedione on the aldosterone mediated transactivation of the hMR.

Comparison	Mean difference	t	P < 0.05?
250 nmol/l vs 100 nmol/l	-0,1522	1,264	No
250 nmol/l vs 50 nmol/l	-0,3702	3,073	No
250 nmol/l vs 25 nmol/l	-0,2441	2,027	No
250 nmol/l vs 10 nmol/l	-0,1788	1,484	No
250 nmol/l vs 1 nmol/l	-0,1100	0,9129	No
250 nmol/l vs 0 nmol/l	-0,01177	0,09770	No
100 nmol/l vs 50 nmol/l	-0,2180	1,810	No
100 nmol/l vs 25 nmol/l	-0,09192	0,7631	No
100 nmol/l vs 10 nmol/l	-0,02654	0,2204	No
100 nmol/l vs 1 nmol/l	0,04224	0,3507	No
100 nmol/l vs 0 nmol/l	0,1404	1,166	No
50 nmol/l vs 25 nmol/l	0,1261	1,047	No
50 nmol/l vs 10 nmol/l	0,1915	1,589	No
50 nmol/l vs 1 nmol/l	0,2602	2,160	No
50 nmol/l vs 0 nmol/l	0,3584	2,976	No
25 nmol/l vs 10 nmol/l	0,06538	0,5427	No
25 nmol/l vs 1 nmol/l	0,1342	1,114	No
25 nmol/l vs 0 nmol/l	0,2324	1,929	No
10 nmol/l vs 1 nmol/l	0,06878	0,5710	No
10 nmol/l vs 0 nmol/l	0,1670	1,386	No
1 nmol/l vs 0 nmol/l	0,09820	0,8152	No

Part III

Discussion and summary

Chapter 9

General discussion and future perspectives

Since the first case of congenital adrenal hyperplasia (CAH) was described in 1865 [1], a vast amount of knowledge on the pathophysiology, clinical features and treatment of CAH has been gathered. However, there are still gaps in our knowledge and new questions have emerged. This thesis aims to add new insights to the existing knowledge on CAH. In the first part of the thesis, we describe patterns of linear growth and bone maturation in untreated nonclassic (NC) CAH patients and treated classic CAH patients, as well as salivary steroid patterns in treated classic CAH patients. In the second part of the thesis, we focus on the effect of adrenal steroid precursors that aberrantly accumulate in CAH patients on the glucocorticoid and mineralocorticoid receptor. In this concluding chapter, the results described in this thesis are discussed in view of current literature. The clinical implications of our findings are discussed and recommendations for future research are made.

Patterns of growth and salivary steroid levels in CAH patients

Growth patterns in CAH

It has been well established that monitoring of linear growth is an important aspect of the clinical care of pediatric CAH patients. [2] Untreated or poorly treated patients are exposed to increased levels of adrenal androgens, resulting in an increased growth velocity, an advanced bone age with early epiphyseal maturation, and eventually a decreased final height. However, treatment with (supraphysiological doses of) glucocorticoids can also result in a diminished final height due to a reduction of growth hormone secretion and action, disturbances of the calcium metabolism and direct effects on the growth plate. [2-4] Therefore, keeping the balance between overtreatment and undertreatment is the main challenge for pediatric endocrinologists taking care of CAH children.

Final height in CAH patients has been studied previously, and two meta-analyses have been published. [5, 6] Both report a decreased final height compared to the general population and compared to midparental height. However, the studies included in the meta-analyses suffer from serious methodological issues and there is a large inconsistency of results across included studies, resulting in a low level of evidence. Among these studies, there is large variability in severity of CAH, in monitoring regimen, in glucocorticoid treatment and in the patient categories that are included. Incidentally, predicted final height is used instead of actual final height and in a number of studies parental height is not available. Secular trend is usually not considered, which may result in an underestimation of the expected final height. [5, 6] Studies on the determining factors of final height are further

complicated by the long term follow-up that needs to be evaluated, by the influence of both the disease and the treatment on growth, and by the low incidence of CAH. The final height that we have found in classic CAH patients seems more decreased than reported in the two meta-analyses, -1.63 SDS versus -1,21 SD and -1,02 SD. This might partially be explained by the fact that we do take secular trend into account and thus do not underestimate target height. Also, we made a strict selection of patients to ensure the study group is a homogenous group of severe CAH patients.

Both studies on growth patterns in CAH included in this thesis illustrate that merely describing linear growth is not sufficient. The final height is determined by the balance between growth velocity and bone maturation. When growth velocity is increased, but the advancement of bone maturation is more severe, final height will be reduced. When the increased growth velocity and advanced bone maturation keep equal pace, final height may not be reduced. Most NC-CAH children seem to reach a final height within their target range [7] and glucocorticoid treatment is not necessarily indicated in NC-CAH children to improve their final height. [2, 7-10] However, we have shown that in NC-CAH patients an increased growth velocity can be absent while bone maturation is advanced. Both height velocity and bone age should be taken into account by clinicians considering treatment, weighing the risk of not treating and the risk of growth suppression due to glucocorticoid treatment.

Previously it has been concluded that in classic CAH children mainly the pubertal growth is reduced, resulting in a decreased final height. [11-13] However, our evaluation of linear growth as well as bone maturation in classic CAH patients reveals that the loss of growth potential already occurs in early childhood (see further). Establishing the diagnosis of CAH as early as possible is crucial to lower the exposure to adrenal androgens by early glucocorticoid treatment, to improve final height outcome. [5, 14] Since the patients in our study – as well as the patients reported in other studies – were diagnosed before the introduction of national neonatal screening programs and were therefore not all treated from a young age, we expect that final height outcomes in currently treated pediatric CAH patients will improve. Neonatal screening for CAH was implemented in the Netherlands in 2002, so the first patients diagnosed in the screening program are now reaching their final height. This creates opportunities for new evaluations of growth patterns in CAH children. Longitudinal, multi-center registries could be of great value to overcome some of the difficulties in studying long term outcomes mentioned previously.

Age-specific treatment of CAH

Nowadays, almost all CAH patients reach adulthood with a good quality of life. However, both undertreatment with exposure to increased levels of androgens, and overtreatment with exposure to supraphysiological amounts of glucocorticoids are common. The reduced final height in which this may result is a reliable long term outcome measure for adequate treatment. Although the effect of a decreased final height on the quality of life is not clear [6], there are other potential long term complications from both under- and overtreatment that are worth avoiding. For example, prolonged exposure to increased levels of androgens can result in gonadal dysfunction and fertility problems in both male and female CAH patients. [15-17] Long term treatment with supraphysiological doses of glucocorticoids can result in side effects such as hypertension, obesity and a reduced bone mineral density. [16, 18] Therefore, studies on growth patterns in CAH patients can yield recommendations on CAH treatment that are relevant for more than height alone. Although not all previous studies have found a correlation between glucocorticoid treatment and height outcomes, our results and those of other groups do, yielding age-specific treatment suggestions. [6, 14] Infancy, childhood and puberty should be regarded as distinct periods, each with their own treatment goals and approaches (Figure 1).

In **infancy** (0 – 1 year), patients are relatively insensitive to the effects of androgens on growth. [14, 19] However, higher doses of glucocorticoids may already have

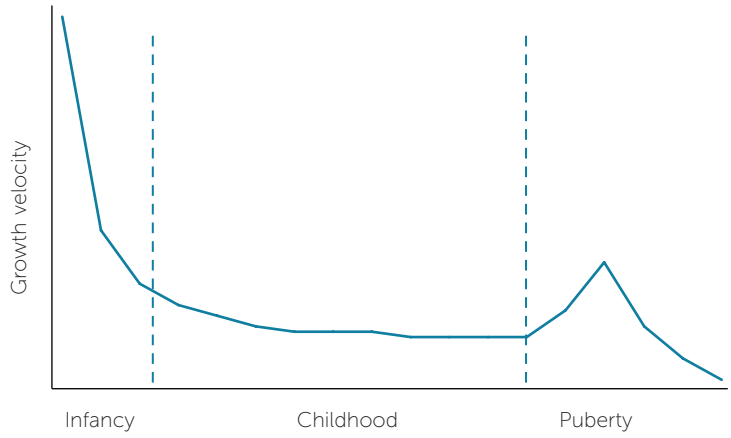


Figure 1 Growth velocity during infancy, childhood and puberty.

Treatment and treatment goals of CAH patients may be different in these periods, as growth velocity, sensitivity to androgens and sensitivity to glucocorticoids may change.

deleterious effects on growth because of the high growth velocity in infants. Therefore, it can be advised that in infancy glucocorticoid overtreatment should be especially avoided and patients should be treated with physiological doses of glucocorticoids. However, as yet longitudinal studies confirming an improved outcome with this treatment strategy are lacking. Hopefully, longitudinal registries will provide us with these data in the future.

In **childhood** (age 1 year until the onset of puberty), patients seem to become more sensitive to the androgen-related effects on growth and maturation. Strict monitoring of androgen levels and appropriate hydrocortisone dose adjustments are indicated to avoid exposure to increased androgen levels and accelerated bone maturation. Supraphysiological doses of hydrocortisone may be necessary in this period, but the dose should be as low as possible, adequately suppressing androgen production while avoiding overtreatment. [2, 9] Adequate monitoring of treatment is crucial to detect signs of under- or overtreatment at an early stage.

Puberty might be the most challenging phase in the treatment of CAH patients. The growth velocity increases again, due to the effect of the sex steroids. Glucocorticoid metabolism changes, with an increased cortisol clearance due to decreased activity of 11β-hydroxysteroid dehydrogenase type 1. [20, 21] Also, decreased insulin sensitivity can result in increased adrenal and ovarian androgen synthesis. [14, 22] Furthermore, adherence to medical treatment may be more complicated due to psychosocial issues. [22] Based on our studies, showing that in puberty increased doses of glucocorticoids were associated with a decreased final height, we suggest that glucocorticoid doses should not be routinely increased in puberty despite the increased cortisol clearance, to avoid overtreatment. The patients should be treated with the lowest possible glucocorticoid dose, possibly allowing adrenal androgen levels to be slightly increased. [2, 12, 22]

Besides these age-specific aspects, individual patient characteristics can influence the effect of glucocorticoid treatment. Individual variation in absorption from the gastro-intestinal tract, corticosteroid binding globulin concentrations and cortisol half-life in the circulation influence optimal dosing. [23] Glucocorticoid receptor polymorphisms might results in variations in individual glucocorticoid sensitivity. [24] Also, complications arising in CAH patients can make adaptations in treatment necessary. For example, when adolescent males develop testicular adrenal rest tumors, or when adolescent females suffer from menstrual irregularity due to slightly elevated adrenal progestins, it can be appropriate to increase the glucocorticoid dose. [17, 25, 26] The benefits and risks of adjusting the glucocorticoid dose should continuously be weighed, treatment should be monitored carefully, and the treatment should be individualized as much as possible.

It is questionable whether optimally individualized and age-specific treatment can be achieved with the currently common treatment modalities. Current recommendations are to treat pediatric CAH patients with hydrocortisone, 10-15 mg/m^2/day in three daily doses.[27] Long-acting glucocorticoids such as prednisolone and dexamethasone are generally avoided in children, because of the suspected higher risk of growth suppression. [2, 27] There is still controversy on whether the highest dose of hydrocortisone should be given in the evening, with the goal of optimally suppressing the hypothalamic-pituitary-adrenal axis at its peak activity, or in the morning, mimicking the normal diurnal rhythm. [28]

Several new treatment strategies are being explored, with the hopes of coming closer to achieve the much-needed balance between appropriate suppression of adrenal androgens and not exposing patients to supraphysiological amounts of glucocorticoids. [27, 29] Improvements in the delivery and dosing schedule of glucocorticoids are investigated, for example using modified release oral glucocorticoids and continuous subcutaneous hydrocortisone pumps. [30-32] Also, attempts are made to find nonglucocorticoid strategies to diminish androgen excess, such as androgen biosynthesis inhibitors and corticotrophin releasing hormone receptor antagonists. [33, 34] Data on the long term effects and on the results in pediatric CAH patients are not yet available, but hopefully these attempts will provide new modalities for optimal and individualized treatment of CAH patients in the future.

Salivary steroid measurements

Whichever treatment strategy is chosen, monitoring of the treatment is of utmost importance. Both clinical and biochemical parameters should be used in the long term monitoring of CAH patients. [2, 9, 22, 27] Unfortunately, current monitoring strategies are far from perfect. Growth velocity and bone age reflect androgen exposure, however the cumulative effect of exposure to androgens over time is difficult to estimate. Periodic measurements of 17-hydroxyprogesterone (17OHP) and androstenedione (A), the most commonly used biochemical parameters, only reflect short term hormonal control. Taking this into account, salivary steroid measurements can be of great value in clinical practice for the monitoring of adrenal steroid precursor levels. The salivary levels of 17OHP and A reliably represent serum levels. [35, 36] Collection of saliva is stress-free. Patients can collect multiple samples per day before taking their glucocorticoid medication, reflecting the diurnal rhythm of steroid synthesis and reliably representing endogenous steroid production. Saliva collection can be performed at home and the samples can be sent to the hospital by mail, ensuring accessibility of this monitoring method to all patients.

Unfortunately, saliva collection is difficult in young children, who are not able to collect the saliva on command. As described previously, in young children after

infancy adequate monitoring of adrenal androgen levels and adequate treatment are as important as in older children. Therefore, it would be desirable to develop novel methods for saliva collection in young children.

Current treatment goals are to suppress the A levels to within age- and sex appropriate reference values and to not completely suppress 17OHP levels. [2, 27] It can be questioned whether these goals are specific enough, and whether they remain constant throughout childhood. It can even be debated whether 17OHP and A are the best markers for adequate disease control. 21-deoxycortisol (21DF) might prove to be a more reliable parameter in the future (see further). Potentially, measurement of cortisol and steroid precursors in scalp hair could be used to evaluate steroid levels over a longer period of time. [37, 38] Further studies are needed to determine its value in clinical practice.

It needs to be noticed that no single biochemical parameter can suffice to monitor the treatment of CAH patients. The entire clinical picture should always be taken into account.

Glucocorticoid and mineralocorticoid action of steroid hormone precursors

Glucocorticoid activity of adrenal steroid hormone precursors

It has been well established that adrenal steroid hormones can cross-activate the human glucocorticoid receptor (hGR) and the human mineralocorticoid receptor (hMR). The adrenal steroid hormones and their precursors are structurally similar, and the DNA binding domain of the hGR and the hMR is highly homologous. [39] It has been shown previously that aldosterone and cortisol can each activate both receptors. [40-43] Also, progesterone and 17OHP are known to activate the hGR. [43-45] We have further expanded this knowledge, by evaluating the ability of more than ten adrenal steroid precursors to transactivate the hGR and determine their relative glucocorticoid potency to cortisol. We have shown that mainly 21DF and corticosterone, and to a lesser extent 11-hydroxyprogesterone and 11-deoxycortisol, are able to transactivate the hGR.

21-deoxycortisol

Of all steroid precursors, 21DF deserves special attention. It is formed when 17OHP is 11β-hydroxylated. In healthy subjects, 21-hydroxylation of 17OHP is the preferred pathway and 21DF levels are very low. However, in CAH patients 17OHP cannot be 21-hydroxylated and the excess 17OHP is converted to 21DF. [46, 47]

21DF has been shown to have relatively high glucocorticoid potency, close to cortisol itself. Furthermore, it is metabolized via the 'backdoor' pathway to steroids with potent androgenic activity. [48]

Because of the increased levels of 21DF specifically in CAH patients, there may be a role for it in clinical practice. 21DF can be of added value in the diagnostic process of CAH, for example in newborn screening, and may be more specific than 17OHP. [9, 49-51] Also, in contrast to 17OHP, the levels of 21DF do not change with either puberty or the menstrual cycle since 11β-hydroxylation is restricted to the adrenal cortex. [46, 47, 52, 53] Thus, suppression of 21DF may be a more reliable target for glucocorticoid treatment than 17OHP. However, clinically relevant target levels need to be determined.

Clinical implications of glucocorticoid activity of adrenal steroid precursors

As described previously, 21DF and several other steroid hormone precursors that are present in increased levels in CAH patients, have glucocorticoid potency *in vitro*. Besides increasing the understanding of pathophysiological mechanisms, there may be several clinical consequences relevant for CAH patients. It seems that the elevated levels of adrenal steroid precursors may at least partially compensate for cortisol deficiency. This protective mechanism could explain the lack of signs of cortisol deficiency we have encountered in the patients described in chapters 5 and 7. Also, it might explain the rarity of signs of adrenal insufficiency in NC-CAH patients. [54]

It should be noted, that the possible protective effects of elevated adrenal steroid precursors disappear once glucocorticoid treatment is initiated. Due to the glucocorticoid treatment, ACTH and consequently the adrenal steroid precursors are suppressed. Thus, once maintenance-therapy is started, adequate stress-dosing is of utmost importance. Recent studies have shown, that almost all episodes of adrenal insufficiency and deaths in treated CAH patients have been preceded by a lack of applying adequate stress-dosing. [55, 56]

The implications of the glucocorticoid activity of elevated levels of adrenal steroid precursors for clinical practice are still unclear. Based on current knowledge it cannot be determined to what extent individual patients are protected from developing clinical signs of adrenal insufficiency by elevated steroid precursors. We have shown that some untreated CAH patients with severe enzyme deficiencies can survive until adulthood, but we cannot predict which patients this will be.

Hypothetically, the glucocorticoid potency of elevated adrenal precursors may have treatment implications for two patient categories: classic CAH patients in areas with limited resources, and non-classic CAH patients.

1. When local knowledge, education opportunities, resources and infrastructure are insufficient to guarantee access to adequate glucocorticoid medication and stress-dosing, it may be safer for a subset of CAH patients not to be treated with glucocorticoids at all, as treatment will suppress the levels of protective steroid precursors, especially when only long-acting steroids are available. When stress-dosing is not applied adequately, or when maintenance treatment is discontinued, the risk of developing adrenal crises might be increased compared to untreated patients. However, we are not yet able to predict which patients belong to this subset. Also, in untreated CAH patients there will be signs of hyperandrogenism and possible long term complications of exposure to increased levels of adrenal androgens that should be considered. Alternative strategies focusing on decreasing the androgen secretion and/or action might be of use in these patients.
2. The discussion on the need of stress-dosing in untreated NC-CAH patients is ongoing. It has been reported that in 60% of NC-CAH patients, the response of cortisol to tetracosactide is suboptimal, suggesting a potential risk of adrenal crises. However, clinical signs of adrenal insufficiency seem uncommon. [54] When the adrenal precursors with glucocorticoid activity are sufficiently elevated, this could potentially be an additional argument to withhold stress-dosing in these untreated patients.

To determine whether untreated CAH patients are adequately protected from cortisol deficiency by the accumulated adrenal steroid precursors, the standard ACTH-stimulation test is not sufficient. Ideally, the ACTH-stimulated levels of all relevant adrenal steroid precursors should be considered, and weighed for their relative glucocorticoid potency. Additionally, adequate reference values need to be determined. Even for the routinely used ACTH stimulation test, no agreement exists on the appropriate cut-off levels for maximal serum cortisol response. [57, 58] For the combined glucocorticoid activity of all steroids and steroid precursors, clinically relevant cut-off levels will need to be established to be of use in daily practice.

Thus, combined steroid profiling with adequately determined cut-off levels could be of great value in making treatment decisions. However, more translational research will be required to develop this glucocorticoid activity tool.

Mineralocorticoid activity of adrenal steroid hormone precursors

In contrast to the glucocorticoid potency of several adrenal steroid hormone precursors, it has been shown that 17OHP and progesterone have anti-mineralo-

corticoid potency *in vitro*. This can be of clinical relevance in CAH patients as well. In untreated CAH patients, salt wasting can be exaggerated, when the remaining aldosterone is counteracted by the elevated steroid precursors. Furthermore, neonates are relatively resistant to aldosterone. Thus, during the neonatal period higher doses of mineralocorticoids and sodium chloride may be required to relieve salt wasting. Once the glucocorticoid treatment adequately suppresses the adrenal steroid precursor levels, the need for mineralocorticoids may decrease and potentially the dose can be lowered to prevent overtreatment, which is associated with complications such as hypertension. Monitoring of renin levels and blood pressure can aid the clinician in adequately dosing mineralocorticoid treatment.

General conclusion – one size does not fit all

In this thesis we have explored several consequences of the elevated levels of adrenal steroid precursors in CAH patients. We have described the patterns of salivary steroid levels in childhood and adolescence, and the consequences for linear growth and bone maturation. We have shown the potential glucocorticoid and anti-mineralocorticoid effects of adrenal steroid precursors, and the clinical consequences this may have for CAH patients.

Overall it can be concluded that the care of CAH patients cannot be based on a 'one size fits all' principle. Long term outcome is determined by many different factors (Figure 2). Monitoring and treatment decisions should be tailored to the individual patient's needs and characteristics. Diverse aspects such as severity of the enzymatic defect, stage of life, but also local availability of medical resources should be considered.

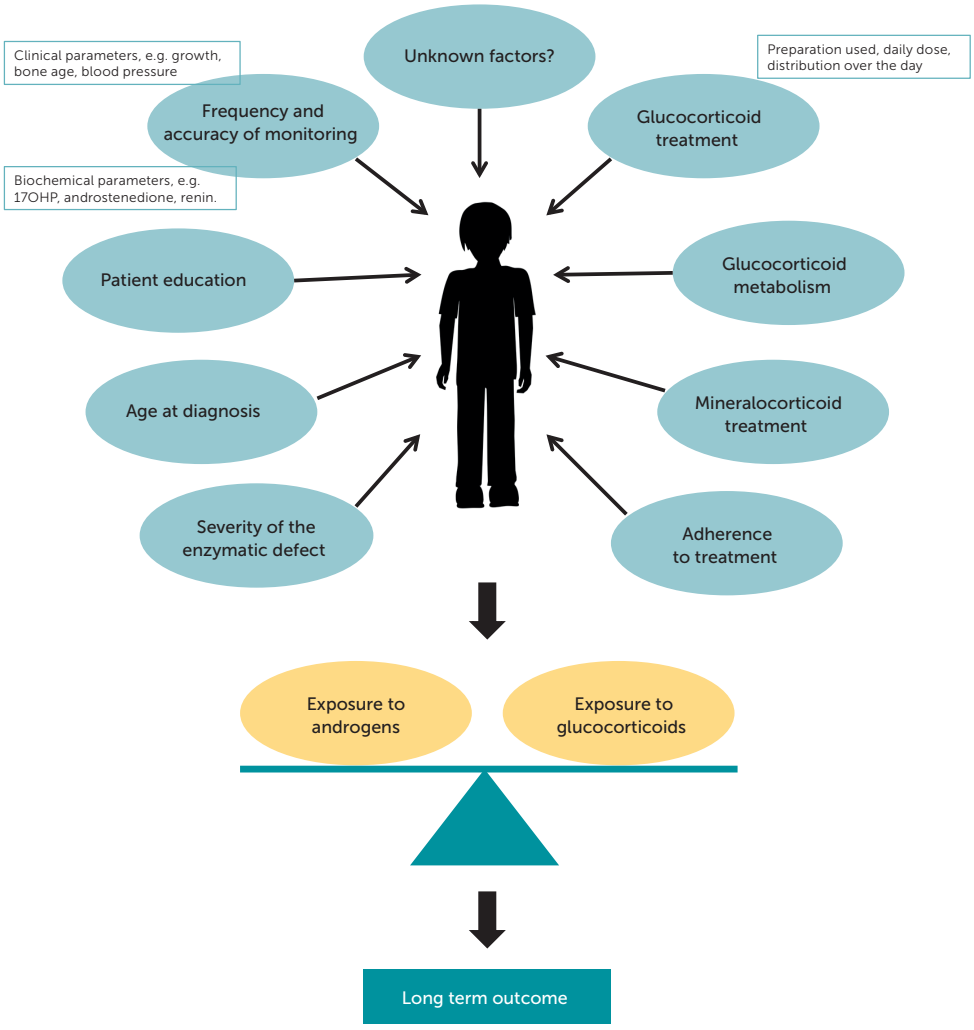


Figure 2 Factors influencing long term outcome in pediatric CAH patients.

Long term outcome is determined by the balance between exposure to adrenal androgens and exposure to glucocorticoids. This balance is influenced by multiple factors.

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Chapter 10

Summary

The studies described in this thesis aim to provide more insight in the pathophysiological processes related to the elevated levels of adrenal steroid precursors in congenital adrenal hyperplasia (CAH) patients. In the first part of the thesis (chapter 2-4), the perspective is mostly clinical and the focus lies on growth patterns and salivary steroid levels in CAH patients. The studies in the second part of the thesis (chapter 5-8) focus on the glucocorticoid and mineralocorticoid potency of accumulating adrenal steroid precursors. This chapter summarizes the studies and their most important outcomes.

Chapter 2 describes growth patterns of nonclassic (NC) CAH children. In classic CAH, elevated concentrations of adrenal androgens lead to accelerated growth and bone maturation with compromised adult height. However, in children with NC-CAH, the concentrations of adrenal androgens are generally only slightly increased. We conducted a retrospective study to describe the growth and bone maturation in untreated NC-CAH children to determine the effect of these only slightly elevated adrenal androgen levels. 24 patients were included and divided in a symptomatic (n=17) and an asymptomatic (n=7) group. In the symptomatic patients, height SDS corrected for target height (HSDS-THSDS) only slightly increased with 0.06 SDS per year (95% CI 0.02 – 0.10). Mean bone age advancement (BA_C) was 2.21 years (SDS 0.66, $p< 0.0001$). In asymptomatic patients no significant growth acceleration or BA_C was found. Therefore we concluded that in untreated NC-CAH children growth acceleration is small and generally not visible in their growth charts. BA_C can be more pronounced. Therefore, the absence of an increase in growth velocity does not exclude the diagnosis NC-CAH. When considering this diagnosis and when monitoring NC-CAH patients over time, bone age acceleration should be taken into account.

In **chapter 3**, we describe the salivary levels of 17-hydroxyprogesterone (17OHP) and androstenedione (A) in classic CAH patients, both in childhood and in puberty. Monitoring of treatment by measurement of these adrenal steroid precursors in saliva is a patient friendly and reliable method. However, there are no objective criteria for setting relevant target values or data on changes of 17OHP and A during monitoring. Therefore, we evaluated A and 17OHP levels in nearly 2000 salivary samples collected during long term treatment of 84 pediatric patients with classic 21-hydroxylase deficiency.

17OHP and A levels, and its ratio 17OHP/A, remained constant from the ages of 4 to 11 years with no sex-related differences. During puberty, 17OHP and A levels both increased, starting at earlier age in girls than in boys reflecting the earlier start of puberty in girls. A normalised A level concomitant with an elevated 17OHP level could be obtained with moderate glucocorticoid dosages of 11 – 15 mg/m^2/day.

A levels above the upper reference limit (URL), suggesting undertreatment, coincided with 17OHP levels ≥10 times URL. The percentage of A levels above URL increased in puberty, especially in boys.

In **chapter 4** we describe the final height (FH) of classic CAH children, and age-specific factors that contribute to the FH. FH is a relevant long term outcome measure in CAH patients, since both over- and undertreatment with glucocorticoids can negatively influence FH.

We retrospectively evaluated longitudinal data of 39 pediatric CAH patients. We analyzed height and bone age at diagnosis or at 4 years of age, at the start of puberty, and at FH. Height data were corrected for parental height and secular trend. Hydrocortisone use and salivary steroid levels were studied longitudinally throughout childhood and puberty.

We found that median FHSDS corrected for target height SDS (THSDS) was decreased, at -1.63 SDS. FH was most compromised in simple virilizing (SV) CAH patients (FHSDS–THSDS -1.92 in males, -1.93 in females), while FH in salt wasting (SW) CAH patients was closer to their predicted final height (FHSDS–THSDS -1.27 in males and -0.79 in females). Both in SV and in SW-CAH patients, median height SDS corrected for THSDS decreased from diagnosis or the age of four years to FH. However, when height was corrected for bone age, no height loss occurred from diagnosis or age 4 years to FH in any of the subgroups. Therefore, we conclude that although the height loss only becomes visible in the growth charts in the course of puberty, the loss of growth potential already occurs in early childhood.

Hydrocortisone dose and FH were negatively associated throughout childhood and puberty. However, in the combined model with A, in childhood only A levels were negatively associated with FH. Thus, in prepubertal children, the most important factor contributing to a decreased final height is exposure to elevated androgen levels. In puberty, the growth suppressing effects of hydrocortisone outweigh the negative effects of elevated androgen levels. Therefore, we suggest that the treatment goals should be more specified according to age to find the optimal balance in growth and maturation.

Chapter 5 contains a case description of a patient with SW-CAH, in whom the diagnosis and treatment were delayed for several years. Due to the co-incidence of posterior urethral valves and an initial false diagnosis of secondary pseudohypoaldosteronism explaining his hyponatremia, he was not treated with glucocorticoids or mineralocorticoids for the first two years of life. He did not develop signs of cortisol deficiency despite undergoing surgery and suffering several illnesses. Based on these and other clinical observations, we hypothesize that in untreated CAH patients elevated levels of steroid precursors may partially compensate cortisol deficiency.

This hypothesis is further explored in **chapter 6** by studying the *in vitro* effects of adrenal steroid precursors on the human glucocorticoid receptor (hGR): the binding, nuclear translocation and finally transactivation of the hGR when exposed to 17OHP, A, progesterone and 21-deoxycortisol (21DF). Competitive binding assays were performed in HeLa cells. 17OHP, progesterone and 21DF are able to bind to the hGR with binding affinities of 24 – 43% compared to cortisol. A has a low binding affinity. Nuclear translocation of the hGR was studied by transfection of COS-7 cells with a GFP-tagged hGR and fluorescence microscopy. Incubation with 21DF led to complete nuclear translocation of the hGR, whereas treatment with 17OHP or progesterone resulted in partial nuclear translocation.

Transactivation assays were performed in COS-7 cells and in HEK293 cells after co-transfection with hGR and luciferase reporter vectors using a dual luciferase assay. 21DF transactivated the hGR with an EC50 approximately 6-fold the EC50 of cortisol. 17OHP and progesterone transactivated the hGR with EC50s of more than 100 times the EC50 of cortisol. No hGR transactivation was detected after incubation with A. In conclusion, 21DF, 17OHP and progesterone are able to bind, translocate and transactivate the hGR *in vitro* and thus may have glucocorticoid activity. Mainly 21DF might have a clinically relevant agonistic effect on the hGR and could potentially partially compensate the cortisol deficiency in CAH patients.

In **chapter 7**, the research on the glucocorticoid potency of adrenal steroid precursors is continued. Previous results are replicated, the transactivation of the hGR by previously not studied steroid precursors is evaluated, and clinical and biochemical data of untreated classic CAH patients are described.

We describe a unique population of 22 severely affected untreated CAH patients (age 0 - 46 years), with proven cortisol deficiency (<500nmol/L after ACTH stimulation) but without clinical signs of cortisol deficiency even in situations of severe stress.

Blood concentrations of adrenal steroid precursors that aberrantly accumulate due to the enzymatic defect were assessed by LC-MS/MS. The levels of the steroid precursors 11-deoxycortisol, 11-deoxycorticosterone, 17OHP, 21DF and progesterone were significantly elevated compared to a control group.

The effect of progesterone, 17-hydroxypregnenolone, pregnenolone, aldosterone, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, 17OHP, 11β-hydroxy-progesterone, 16-hydroxyprogesterone and 21DF on the hGR transactivation was evaluated *in vitro*. The hGR was activated with comparable potency to cortisol by 21DF, and 4-100x lower potency by the other measured adrenal steroid precursors.

In conclusion, we identified strongly elevated adrenal steroid precursor concentrations in blood from untreated CAH patients and demonstrated glucocorticoid activity of these adrenal precursors *in vitro* – implicating that these precursors

might be able to partially compensate for cortisol deficiency in untreated CAH patients.

In **chapter 8**, the effect of adrenal steroid precursors on the human mineralocorticoid receptor (hMR) is described. *In vitro* transactivation assays of COS7 cell transfected with the hMR were performed, to assess the mineralocorticoid potency of 17OHP, progesterone, A and testosterone. By exposure of the hMR to aldosterone in combination with the adrenal steroid precursors it was shown that transactivation of the receptor was inhibited by 17OHP and progesterone, with a linear increase of inhibition with increasing concentrations of these steroids. A and testosterone did not affect transactivation of the hMR by aldosterone. In conclusion, while the glucocorticoid potency of accumulating steroid precursors in CAH patients may have a protective effect, elevated concentrations of 17OHP and progesterone have an anti-mineralocorticoid effect *in vitro* that may clinically contribute to an increase of salt wasting and a higher requirement of mineralocorticoids in poorly controlled CAH patients.

Chapter 9 discusses the findings in our studies in view of current literature, and elaborates on possible clinical implications of our results. We provide a general conclusion and give suggestions for future research.

Addendum

Nederlandse samenvatting
Dankwoord
Curriculum Vitae
List of publications
List of abbreviations

Samenvatting

De studies die opgenomen zijn in dit proefschrift hebben tot doel om meer inzicht te verkrijgen in enkele klinische en pathofysiologische processen bij patiënten met het adrenogenitaal syndroom (AGS). In het eerste deel van het proefschrift (hoofdstuk 2-4) ligt de nadruk op de lengtegroei van AGS patiënten, en op de rol van steroïdhormonen gemeten in het speeksel. De studies in het tweede deel van het proefschrift (hoofdstuk 5-8) richten zich op de glucocorticoïde en mineralocorticoïde effecten van de verhoogde concentraties steroïdvoorlopers, die karakteristiek zijn voor AGS. Dit hoofdstuk biedt een samenvatting van de studies en hun belangrijkste resultaten.

Hoofdstuk 1 geeft een overzicht van de fysiologie van de bijnier, en van het ziektebeeld AGS. In de bijnierschors worden vanuit de gezamenlijke bron cholesterol verschillende steroïdhormonen gemaakt: glucocorticoïden (cortisol), mineralocorticoïden (aldosteron) en androgenen. De productie van cortisol wordt gereguleerd door de hypothalamus-hypofyse-bijnier as. CRH uit de hypothalamus stimuleert de afgifte van ACTH door de hypofyse. ACTH stimuleert op zijn beurt de bijnier om cortisol te produceren. Door middel van een nauwkeurig afgesteld terugkoppelingssysteem, waarbij cortisol de afgifte van CRH en ACTH remt, blijft het systeem in balans.

Om in de bijnier vanuit cholesterol de steroïdhormonen te vormen zijn verschillende enzymatische stappen nodig. Bij AGS is er een tekort aan één van de enzymen die nodig zijn voor de vorming van de bijniersteroïden, meestal 21-hydroxylase. Hierdoor is er een tekort aan cortisol en vaak ook aan aldosteron. Hierdoor ontbreekt de negatieve terugkoppeling naar de hypofyse en wordt er steeds meer ACTH geproduceerd om de bijnier te stimuleren. Daardoor worden verhoogde hoeveelheden van voorlopersteroïden (precursors) gevormd, die niet verder omgezet kunnen worden tot cortisol en aldosteron. De verhoging van deze precursors, met name 17-hydroxyprogesteron (17OHP) en androsteendion (A), is karakteristiek voor AGS patiënten en kan gemeten worden in onder meer bloed en speeksel. Deze voorlopersteroïden kunnen door het enzymtekort niet of slechts gedeeltelijk omgezet worden in cortisol en aldosteron, maar wel in bijnierandrogenen. Het tekort aan cortisol kan leiden tot een Addisonse crisis. Het gebrek aan aldosteron leidt tot renaal zoutverlies. Het overschot aan androgenen leidt tot virilisatie van het uitwendig genitaal bij meisjes, wat al antenataal kan optreden.

Er zijn verschillende vormen van AGS, afhankelijk van de ernst van het enzymtekort: klassiek AGS, dat onderverdeeld kan worden in een zoutverliezende (salt wasting, SW) en een niet-zoutverliezende (simple virilizing, SV) vorm, en het mildere, niet klassieke AGS (nonclassic, NC).

Patiënten met klassiek AGS worden behandeld met glucocorticoïden (hydrocortison) en indien nodig ook mineralocorticoïden (fludrocortison). Hierdoor worden niet alleen de tekorten aangevuld, maar wordt ook de negatieve terugkoppeling naar de hypofyse hersteld. De stimulatie van de bijnier door ACTH neemt af, waardoor ook de overmatige productie van androgenen daalt. Patiënten met NC-AGS worden meestal alleen behandeld bij klinisch relevante tekenen van hyperandrogenisme.

In **hoofdstuk 2** worden de groeipatronen van kinderen met de NC vorm van AGS beschreven. Het is bekend dat de overmaat aan bijnierandrogenen bij kinderen met klassiek AGS leidt tot een versnelling van de lengtegroei, maar ook tot een versnelde botrijping. Hierdoor bereiken deze patiënten uiteindelijk een kleinere lengte. Bij kinderen met NC-AGS zijn de bijnierandrogenen echter slechts mild verhoogd. Wij beschrijven retrospectief de lengtegroei en botrijping van onbehandelde kinderen met NC-AGS, om het effect van deze mild verhoogde androgeenconcentraties te beoordelen.

Bij patiënten mét klinische verschijnselen van AGS (zoals vroegtijdige puberteitskenmerken) was er een minimale groeiversnelling. De botrijping was duidelijk versneld, met een gemiddelde voorsprong in de botleeftijd van ruim 2 jaar. Bij patiënten zonder klinische verschijnselen werd geen groeiversnelling of botleeftijdsvoorsprong gevonden. Wij concludeerden dat bij onbehandelde NC-AGS patiënten de groeiversnelling zo gering is dat deze over het algemeen niet zichtbaar zal zijn in de groeicurve. Wel kan er een uitgesproken voorsprong in de botleeftijd zijn. De afwezigheid van een groeiversnelling sluit de diagnose NC-AGS niet uit. Als deze diagnose overwogen wordt, en wanneer deze patiënten vervolgd worden in de loop van de tijd, dient de ontwikkeling van de botleeftijd meegewogen te worden.

In de studie beschreven in **hoofdstuk 3** worden de concentraties van 17OHP en A in het speeksel van klassieke AGS patiënten geëvalueerd, zowel in de prepuberale fase als in de puberteitsleeftijd. Controle van de instelling van de behandeling door middel van het meten van deze steroïdprecursors is een patiëntvriendelijke en betrouwbare methode. De huidige richtlijnen adviseren om te streven naar concentraties van A in het normale bereik, en om het 17OHP niet volledig te onderdrukken. Er bestaan echter geen objectieve streefwaarden, en er is weinig bekend over de patronen van de concentraties van 17OHP en A gedurende de behandeling. Daarom hebben we de waarden van 17OHP en A geëvalueerd in bijna 2000 speekselmonsters die verzameld zijn tijdens de behandeling van 84 kinderen met klassiek AGS. De waarden van 17OHP en A, en de ratio 17OHP/A bleven constant vanaf de leeftijd van 4 jaar tot de leeftijd van 11 jaar, zonder verschil tussen jongens en meisjes. Tijdens de puberteit stegen de waarden van zowel 17OHP als A. Met doseringen hydrocortison van 11-15 mg/m^2/dag werden normale A-waarden bereikt, met een

niet volledig onderdrukt 17OHP. Het aantal speekselmonsters met A-waarden boven het referentie-interval, passend bij onvoldoende onderdrukking van de bijnierandrogenen, nam toe tijdens de puberteit, en dan met name bij jongens.

Hoofdstuk 4 beschrijft eveneens een onderzoek bij klassieke AGS patiënten. Deze studie was gericht op het evalueren van de eindlengte, en het bepalen van leeftijds-specifieke factoren die hierop van invloed zijn. De eindlengte is een relevante uitkomstparameter in de behandeling van AGS patiënten, aangezien zowel over- als onderbehandeling met glucocorticoïden een negatieve invloed kan hebben op de eindlengte. Het vinden van een goede balans tussen over- en onderbehandeling is een grote uitdaging in de zorg voor patiënten met AGS.

Retrospectief werden longitudinale data van 39 AGS patiënten geëvalueerd. Lengte en botleeftijd bij diagnose, bij het begin van de puberteit en bij het bereiken van de eindlengte werden verzameld. De lengtes werden gecorrigeerd voor de ouderlengtes en voor de seculaire trend. De gebruikte doseringen hydrocortison en de concentraties van 17OHP en A in het speeksel werden longitudinaal geëvalueerd.

Voor de hele groep was de mediane eindlengte gecorrigeerd voor de streeflengte verlaagd, namelijk -1.63 SDS. De eindlengte was het meest verlaagd bij jongens met SV-AGS. Zowel bij SV- als bij SW-AGS patiënten nam de mediane gecorrigeerde lengte af tussen het moment van diagnose en het bereiken van de eindlengte. Als de lengte echter ook werd gecorrigeerd voor de botleeftijd, trad geen verlies van lengte op tussen diagnose en eindlengte. Daaruit concludeerden wij dat hoewel de kleinere gestalte over het algemeen pas in de loop van de puberteit zichtbaar wordt in de groeicurve, het verlies van groeipotentieel al vroeg in de kinderleeftijd ontstaat.

De hydrocortisondosering gedurende de kinderleeftijd en de puberteit was negatief geassocieerd met de eindlengte. Dit gold ook voor de A-concentraties op de kinderleeftijd en de eindlengte, maar niet voor de A-waarden in de puberteit. Vóór de puberteit lijkt daarom de blootstelling aan de verhoogde concentraties van bijnierandrogenen het grootste negatieve effect op de eindlengte te hebben, terwijl in de puberteit het groeiremmende effect van glucocorticoïden de overhand lijkt te krijgen. Daarom adviseren wij om de behandelstrategie meer te specificeren op basis van de levensfase, om de optimale balans in de behandeling te bereiken.

In **Hoofdstuk 5** beschrijven we een patiënt met de zoutverliezende vorm van AGS, bij wie de diagnose pas op de leeftijd van 2 jaar werd gesteld. Neonataal was er sprake van ernstige hyponatriëmie, die werd geduid als passend bij secundair pseudo-hypoaldosteronisme ten gevolge van urethrakleppen. Hiervoor werd hij chirurgisch behandeld en kreeg hij orale zoutsuppletie. Hij werd gedurende zijn eerste twee levensjaren niet behandeld met glucocorticoïden of mineralocorticoïden. Desondanks

ontwikkelde hij geen klinische tekenen van cortisoltekort, terwijl hij wel meerdere episodes van stress doormaakte waarbij te verwachten zou zijn dat een cortisoltekort tot problemen zou leiden, zoals episodes van ziekte en de chirurgische behandeling van zijn urethrakleppen. De diagnose AGS werd pas gesteld toen hij op de leeftijd van 2 jaar nog steeds zoutsuppletie nodig had, en er uitgebreider aanvullend onderzoek werd gedaan.

Naar aanleiding van deze klinische observatie werd de hypothese opgesteld dat de voorlopers van steroïdhormonen, die in verhoogde concentraties voorkomen bij AGS patiënten, mogelijk het cortisoltekort ten dele kunnen compenseren.

Deze hypothese wordt uitgewerkt in **hoofdstuk 6**. Hier beschrijven wij een studie waarin de *in vitro* effecten van de steroïdprecursors op de glucocorticoïdreceptor werden onderzocht. De glucocorticoïdreceptor wordt geactiveerd via verschillende stappen: binding van het ligand, verplaatsing van de receptor naar de celkern (translocatie) en transactivatie. Van de steroïdprecursors 17OHP, A, progesteron en 21-deoxycortisol (21DF) werd onderzocht in hoeverre zij kunnen binden aan de glucocorticoïdreceptor, en in welke mate translocatie en transactivatie van de receptor plaatsvinden.

17OHP, progesteron en 21DF bleken te binden aan de glucocorticoïdreceptor, met bindingsaffiniteiten van 24 – 43% in vergelijking met cortisol (100%). A had echter een lage affiniteit voor de glucocorticoïdreceptor. De nucleaire translocatie van de glucocorticoïdreceptor werd bestudeerd in een cellijn met een glucocorticoïdreceptor gekoppeld aan een fluorescerend eiwit. Blootstelling aan 21DF resulteerde in volledige nucleaire translocatie van de receptor, terwijl incubatie met 17OHP of progesteron leidde tot partiële nucleaire translocatie.

In twee verschillende cellijnen werden transactivatiestudies verricht middels een luciferase assay. 21DF transactiveerde de glucocorticoïdreceptor met een EC50 (de concentratie waarbij 50% van de maximale activatie bereikt wordt) van circa zesmaal de EC50 van cortisol. 17OHP en progesteron transactiveerden de receptor met een EC50 van meer dan 100 keer de EC50 van cortisol. Na incubatie met A trad geen transactivatie op.

Concluderend kunnen 21DF, 17OHP en progesteron binden aan de glucocorticoïdreceptor, waarna nucleaire translocatie en transactivatie kunnen plaatsvinden. Van de onderzochte steroïden heeft 21DF het grootste effect op de glucocorticoïdreceptor. Dit zou klinisch relevant kunnen zijn, en zou mogelijk het cortisoltekort bij AGS patiënten ten dele kunnen compenseren.

In de studie beschreven in **hoofdstuk 7** werd het onderzoek naar de glucocorticoide activiteit van steroïdprecursors voortgezet. Resultaten uit het voorgaande onderzoek werden gerepliceerd, nieuwe steroïden werden toegevoegd en klinische

en biochemische gegevens van onbehandelde klassieke AGS patiënten werden geëvalueerd.

Wij beschreven een uniek cohort van 22 onbehandelde patiënten met ernstige vormen van AGS (leeftijd 0-46 jaar), met bewezen cortisoldeficiëntie maar zonder klinische tekenen van cortisoltekort, zelfs in ernstige stresssituaties. De concentraties van steroïdprecursors uit de bijnier die zich opstapelen als gevolg van de enzymdeficiëntie werden gemeten. De concentraties van de steroïdprecursors 11-deoxycortisol, 11-deoxycorticosteron, 17OHP, 21DF en progesteron waren significant verhoogd in vergelijking met een controlegroep. De concentraties van deze steroïden namen niet verder toe na blootstelling aan ACTH, wat aangeeft dat de bijnier al maximaal gestimuleerd was in een rustsituatie.

De effecten van progesteron, 17-hydroxypregnenolon, pregnenolon, aldosteron, corticosteron, 11-deoxycortisol, 11-deoxycorticosteron, 17OHP, 11β-hydroxyprogesteron, 16-hydroxyprogesteron en 21DF op de transactivatie van de glucocorticoïdreceptor werden *in vitro* onderzocht. 21DF activeerde de glucocorticoïdreceptor in vergelijkbare mate als cortisol, de potentie van de overige gemeten steroïden was 4-100 maal lager.

Concluderend werd vastgesteld dat de concentraties van verschillende steroïdprecursors sterk verhoogd zijn bij onbehandelde AGS patiënten. *In vitro* hebben meerdere steroïdprecursors glucocorticoïde eigenschappen, hoewel in mindere mate dan cortisol. Dit suggereert dat deze voorlopers mogelijk de cortisoldeficiëntie bij AGS patiënten ten dele zouden kunnen compenseren, en deze patiënten zouden kunnen beschermen tegen Addisonse crises.

In **hoofdstuk 8** wordt het *in vitro* effect van de steroïdprecursors op de mineralocorticoïdreceptor onderzocht. *In vitro* transactivatie studies werden verricht na blootstelling van de mineralocorticoïdreceptor aan aldosteron, in combinatie met 17OHP, progesteron, A of testosteron. Transactivatie van de receptor bleek te worden geremd door 17OHP en progesteron. A en testosteron hadden geen invloed op de transactivatie van de glucocorticoïdreceptor.

Concluderend hebben 17OHP en progesteron een antimineralocorticoïde effect *in vitro*, dat bij AGS patiënten zou kunnen leiden tot een toename van het zoutverlies en een hogere behoefte aan mineralocorticoïden.

In **hoofdstuk 9** worden de bevindingen uit deze studies bediscussieerd in de context van de wetenschappelijke literatuur. De mogelijke klinische consequenties van onze resultaten worden besproken. Wij geven een overkoepelende conclusie en doen aanbevelingen voor toekomstig onderzoek.

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Curriculum Vitae

Karijn Jara Kleizen werd op 3 augustus 1983 geboren. Ze groeide op in Doesburg met haar ouders en haar twee zussen. In 2001 behaalde ze haar diploma aan het Stedelijk Gymnasium Arnhem *cum laude*. Van 2001 tot 2007 studeerde zij geneeskunde aan de Radboud Universiteit Nijmegen. Ze behaalde haar propedeuse, doctoraal-diploma en artsexamen *cum laude*. Tijdens haar studie geneeskunde deed zij een wetenschappelijke stage op de afdeling huisartsgeneeskunde van de Sahlgrenska universiteit in Göteborg, Zweden, met als onderwerp 'Motives and experiences of GP's teaching undergraduate medical students'. Voor dit onderzoek ontving zij de studieprijs van de faculteit geneeskunde in 2006.

Van 2007 tot 2009 werkte zij als arts-assistent niet in opleiding op de afdeling kindergeneeskunde van het Radboudumc. In 2009 begon zij met haar specialisatie tot kinderarts. Het eerste deel van deze opleiding vond plaats in het Maxima Medisch Centrum in Veldhoven (opleider: dr. M. de Kleine), waarna zij terugkeerde naar het Radboudumc voor het vervolg van haar specialisatie (opleider: dr. J.M.Th. Draaisma). In deze periode begon zij met haar onderzoek binnen de kinderendocrinologie, in samenwerking met dr. H.L. Claahsen-van der Grinten. Dit onderzoek groeide uit tot een promotietraject. In 2012 ontving zij het Sengers stipendium en een travelling grant van de European Society for Pediatric Endocrinology voor een drie maanden durende onderzoeksstage in het Centre for Endocrinology, Diabetes and Metabolism van de Universiteit Birmingham, Verenigd Koninkrijk.

Nadat zij in 2014 haar opleiding tot kinderarts afrondde, werkte zij gedurende twee jaar als kinderarts in het Canisius Wilhelmina Ziekenhuis in Nijmegen. In 2016 begon zij met haar fellowship kinderendocrinologie in het Radboudumc Amalia Kinderziekenhuis. Zij verwacht dit in oktober 2019 af te ronden. Gedurende deze jaren zette zij haar onderzoek voor dit proefschrift voort. Zij presenteerde haar onderzoek op verschillende nationale en internationale congressen. In 2016 ontving zij de NVE Novartis prijs voor het beste abstract binnen de basale endocrinologie voor 'Glucocorticoid receptor Transactivation by 21-deoxycortisol, 17OHP and progesterone in congenital adrenal hyperplasia'.

In 2011 trouwde zij met Floris Pijnenburg. In 2017 werd hun dochter Vera geboren.

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List of abbreviations

0-9

11OHD	11 hydroxylase deficiency
17β-HSD	17β-hydroxysteroid dehydrogenase
17OHP	17-hydroxyprogesterone
21DF	21-deoxycortisol
21OHD	21 hydroxylase deficiency
3β-HSD	3β-hydroxysteroid dehydrogenase
95%CI	95% confidence interval

A

A	Androstenedione
ACTH	Adrenocorticotropic hormone

B

BA	Bone age
BA_C	Bone age corrected for chronological age
BMI	Body mass index
BSA	Body surface area

C

CA	Chronological age
CAH	Congenital adrenal hyperplasia
COS-7	Fibroblast-like cell line derived from monkey kidney tissue
CRH	Corticotrophin releasing hormone

D

DHEA(S)	Dehydroepiandrosterone(sulphate)

E

EC50	Estimated concentration for 50% transactivation

F

FH	Final height
FHSDS	Final height standard deviation score

G

GFP	Green fluorescent protein

H

HC	Hydrocortisone
HEK293	Human embryonic kidney cell line
HeLa	Cell line derived from cervical cancer from Henrietta Lacks
hGR	Human glucocorticoid receptor
hMR	Human mineralocorticoid receptor
HPA	Hypothalamus-pituitary-adrenal
HSDS	Height standard deviation score

I

IC50	Concentration that reduces binding of a radioligand by 50%
IQR	Interquartile range

L

LC-MS/MS	Liquid chromatography – tandem mass spectrometry

M

MMTV-Luc	Firefly luciferase reporter construct
MWU	Mann Whitney U test

N

NC-CAH	Non classic congenital adrenal hyperplasia

P

P	Progesterone
PCR	Polymerase chain reaction
PHA	Pseudohypoaldosteronism
pRL-TK	Renilla luciferase construct

R

RLU	Relative light units

S

SNP	Single nucleotide polymorphism
SV-CAH	Simple virilizing congenital adrenal hyperplasia
SW-CAH	Salt wasting congenital adrenal hyperplasia

T

TH	Target height
THSDS	Target height standard deviation score

U

URL	Upper reference limit